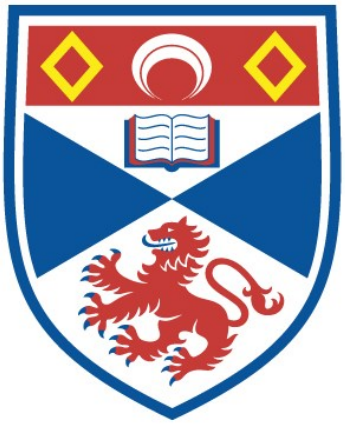
AN EXAMINATION OF NP-P AND NP-V INTERACTIONS WITHIN THE SIMIAN VIRUS 5 (SV5) REPLICATION COMPLEX

Alison Bermingham

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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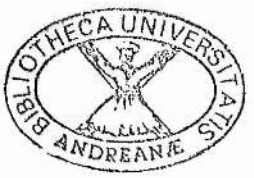

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Science at the University of St. Andrews
November, 1997.

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My thanks must especially go to my mother for proof-reading and not being too disappointed to find that my university education didn't teach me to spell. Without the total support of my family, this thesis would never have been completed.

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ABSTRACT

The aim of this study was to examine the mechanisms of transcription and replication of the paramyxovirus, simian virus type 5 (SV5). This was initially attempted using reverse genetics techniques and subsequently examining specific viral protein : protein interactions within the replication complex.

A cDNA clone encoding a synthetic negative-sense RNA genome analogue was constructed. Reverse genetics techniques were used to attempt to characterise conditions which supported the transcription and replication of this genome analogue, with or without the use of wild-type helper virus but were unsuccessful.

During the course of these studies, a number of mammalian cell lines inducibly expressing SV5 proteins were isolated. These cell lines were subsequently used to examine viral protein : protein interactions within the replication complex.

When expressed alone, both P and V proteins exhibited diffuse cytoplasmic fluorescence and V was also found in the nucleus. However, when NP was expressed alone, it was seen as punctate and granular cytoplasmic fluorescence. The distribution patterns of the proteins changed when expressed in combination. Large cytoplasmic aggregates similar to those at late times in an SV5 infection were seen in cells which co-expressed NP and P. When NP was co-expressed with V, however, NP was partially redistributed to give diffuse cytoplasmic and nuclear fluorescence. This showed that both P and V proteins could interact with NP and suggested that V may play a role in keeping NP soluble prior to an ordered encapsidation process.

Extracts from these cell lines were then used in a novel protein : protein capture assay and demonstrated that NP could interact with both P and V proteins. NP expressed by the cell line was shown to contained both soluble and polymeric forms of NP. P was shown to bind both forms of NP, while V could only bind soluble NP. Since P and V proteins are amino co-terminal, the site of interaction between P and polymeric NP was predicted to be in the P unique C-terminus. This was strengthened when a P-specific C-terminal mAb was found to block the binding of P with polymeric NP. Deletion mutant analysis in the C-terminus of the P protein showed that the mAb binding site was at the extreme C-terminus of the protein suggesting this is the point of interaction between P and polymeric NP. Possible roles for these protein : protein interactions and implications for the paramyxovirus replication complex are discussed.

ABBREVIATIONS

NUCLEIC ACIDS

DNA	2' deoxyribonucleic acid
RNA	ribonucleic acid
A	adenine (base in DNA or RNA)
C	cytosine (base in DNA or RNA)
G	guanine (base in DNA or RNA)
T	thymine (base in DNA)
U	uracil (base in RNA)
NTP(s)	ribonucleoside triphosphate(s)
ATP	adenosine 5' triphosphate
CTP	cytidine 5' triphosphate
GTP	guanosine 5' triphosphate
UTP	uridine 5' triphosphate
dNTP(s)	2' deoxyribonucleoside triphosphate(s)
dATP	2' deoxy-adenosine 5' triphosphate
dCTP	2' deoxy-cytidine 5' triphosphate
dGTP	2' deoxy-guanosine 5' triphosphate
TTP	thymidine 5' triphosphate
ddNTP(s)	2',3' dideoxyribonucleoside triphosphate(s)
ddGTP	2',3' dideoxy-guanosine 5' triphosphate
cDNA	complementary DNA
mRNA	messenger RNA
vRNA	viral genomic or anti-genomic RNA

PHYSICAL UNITS

^{o}C	temperature in degrees Celcius
g	centrifugal force
g	gram mass
mg	milli gram (10^{-3} g)
µg	micro gram (10^{-6} g)
ng	nano gram (10^{-9} g)
l	litre volume
ml	milli litre
µl	micro litre
Ci	Curie (measure of radioactivity=3.7×10^{10} disintegrations per second)
mCi	milli Curie
µCi	micro Curie
M	molar concentration
mM	milli molar
µM	micro molar
kDa	kilodalton
kb	kilobase (pairs)
pH	$-\log_{10}[H^{+}]$
V	volts
mA	milli amperes
U	units of enzyme activity

CHEMICALS AND REAGENTS

^{14}C	radioisotope carbon-14
^{35}S	radioisotope sulphur-35
DAPI	4, 6, diamino-2-phenylindole
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
FITC	fluorescein-isothiocyanate
GMEM	Glasgow modified Eagle's medium
KAc	potassium acetate
NaAz	sodium azide
NBCS	Newborn calf serum
NP40	nonidet p40
PBS	phosphate buffered saline
SDS	sodium dodecyl-sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine tetra-acetic acid
Tris-HCl	tris-hydroxymethyl-aminomethane, pH adjusted with HCl
TE	tris-EDTA

VIRUSES

CDV	canine distemper virus
EMCV	encephalomyocarditis virus
FMDV	foot and mouth disease virus
hPIV1	human parainfluenza virus type 1
hPIV2	human parainfluenza virus type 2
hPIV3	human parainfluenza virus type 3
bPIV3	bovine parainfluenza virus type 3
MeV	measles virus
MuV	mumps virus
NDV	Newcastle disease virus
RSV	respiratory syncytial virus
RV	rabies virus
SeV	Sendai virus
SV5	simian virus type 5
VV	vaccinia virus
VacT7	recombinant VV expressing T7 polymerase
VSV	vesicular stomatitis virus

MISCELLANEOUS

%	percent
% v/v	% volume of total volume
% w/v	% weight of total weight
% w/w	% weight of total weight
BHK	baby hamster kidney (cells)
C-terminus	carboxy terminus
CAT	chloramphenicol acetyl-transferase
DI	defective interfering
E.coli	*Escherichia coli*
ECL	electrochemical light
IF	immunofluorescence
Ig	immunoglobulin
IP	immunoprecipitation
λ	bacteriophage lambda
m.o.i.	multiplicity of infection
mAb	monoclonal antibody
N-terminus	amino terminus
p.f.u.	plaque forming units
p.i.	post infection
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNasin	placental ribonuclease inhibitor
RT	reverse transcription reaction
RT/PCR	coupled RT and PCR reaction
SSPE	subacute sclerosing panencephalitis
TK	thymidine kinase
UV	ultraviolet

GENETIC CODE

TTT phe F	TCT ser S	TAT tyr Y	TGT cys C
TTC phe F	TCC ser S	TAC tyr Y	TGC cys C
TTA leu L	TCA ser S	TAA OCH Z	TGA OPA Z
TTG leu L	TCG ser S	TAG AMB Z	TGG trp W
CTT leu L	CCT pro P	CAT his H	CGT arg R
CTC leu L	CCC pro P	CAC his H	CGC arg R
CTA leu L	CCA pro P	CAA gln Q	CGA arg R
CTG leu L	CCG pro P	CAG gln Q	CGG arg R
ATT ile I	ACT thr T	AAT asn N	AGT ser S
ATC ile I	ACC thr T	AAC asn N	AGC ser S
ATA ile I	ACA thr T	AAA lys K	AGA arg R
ATG met M	ACG thr T	AAG lys K	AGG arg R
GTT val V	GCT ala A	GAT asp D	GGT gly G
GTC val V	GCC ala A	GAC asp D	GGC gly G
GTA val V	GCA ala A	GAA glu E	GGA gly G
GTG val V	GCG ala A	GAG glu E	GGG gly G

AMINO ACIDS

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartate	P	Pro	proline
E	Glu	glutamate	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arganine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

MONOCLONAL ANTIBODIES

The following monoclonal antibodies were used during the course of this study :-

SV5 NP-a	- detects NP in immunofluorescence and immunoprecipitation experiments .
SV5 NP-d	-detects NP in western blots.
SV5 P-k	- 9 amino acid epitope in N-terminal domain common to both P and V proteins. - reacts in western blots, immunoprecipitation and immunofluorescence.
SV5 P-a	- epitope in C-terminal domain of P - reacts in wesern blots and immunofluorescence.
SV5 P-d	- epitope in C-terminal domain of P - reacts in wesern blots and immunofluorescence.
SV5 P-e	- epitope in C-terminal domain of P - reacts in wesern blots and immunofluorescence.

Table of Contents

LIST OF FIGURES

Chapter 1 : INTRODUCTION

This thesis aims to explore the mechanisms involved in the transcription and replication of simian virus 5 (SV5), a member of the paramyxovirus family. These mechanisms were investigated utilising both reverse genetics techniques and by examining specific viral protein : protein interactions within the replication complex. This chapter is therefore divided into three main sections: the first, gives an overview of what is currently known about the *Paramyxoviridae* transcription / replication mechanisms; the second, documents the development of genetic manipulation techniques for the examination of the biology of negative stranded RNA viruses; the third outlines the aims of the project presented in this thesis. Chapter 2 documents the materials and methods employed during the course of this work. The results are presented in Chapter 3 where they have been divided into 3 sections. The first deals with the attempted development of a reverse genetics system for SV5; the second documents the development of cell lines expressing SV5 proteins; the third examines SV5 protein : protein interactions. In Chapter 4, the results, which give some insight into the possible roles played by these proteins in viral transcription and replication, are discussed, with future work also being outlined.

1 Classification, Molecular Structure and Replication of the *Paramyxoviridae*

The *Paramyxoviridae* is a family of enveloped viruses, with a single-stranded, non-segmented, RNA genome of negative polarity. They resemble two other families of negative-stranded RNA viruses, namely the *Rhabdoviridae*, for a similarity of genome organisation and expression, and the *Orthomyxoviridae*, for a similarity in the biological properties of the envelope glycoproteins.

The negative sense RNA genome has a dual purpose: firstly, it is the template for mRNA synthesis, and secondly, is a template for antigenome (full length positive strand copy of the genome) synthesis, which in turn, acts as a template for the synthesis of genomic (negative) sense RNA. These viruses encode their own RNA-dependent RNA polymerase, which acts as both a transcriptase, during the generation of mRNA, and a replicase, when synthesising genomes (or antigenomes). mRNA is generated once the virus has been uncoated in the infected cell and is followed by viral replication, which requires continuous synthesis of viral proteins.

1.1 Classification of the *Paramyxoviridae*

In 1995, the International Committee on Taxonomy of Viruses, reclassified the *Paramyxoviridae* family into 2 subfamilies, namely, the *Paramyxovirinae* and the *Pneumovirinae*. The *Paramyxovirinae* contains three genera, *Parainfluenzavirus, Rubulavirus* and *Morbillivirus*, while the *Pneumovirinae* contains only the *Pneumovirus* genus. This classification was based on morphological similarities, antigenic cross-reactivity between members of a genus, and the coding organisation of the P genes. Examples of viruses found in each genus are given in Table 1. The *Parainfluenzaviruses* and *Rubulaviruses* are differentiated from the *Morbilliviruses* on the basis of biological activity of the attachment protein. In addition, the *Rubulaviruses* contain an extra gene, (SH), which is not seen in the *Parainfluenza-* or *Morbilliviruses*.

Family *Paramyxoviridae*

Subfamily *Paramyxovirinae*

Genus *Parainfluenzavirus*

Sendai virus (mouse parainfluenza virus type 1)

Human parainfluenza virus type 1 and type 3

Bovine parainfluenza virus type 3

Genus *Rubulavirus*

Simian virus 5 (Canine parainfluenza virus type 2)

Mumps virus

Newcastle disease virus

Human parainfluenza virus type 2, type 4a and 4b

Genus *Morbillivirus*

Measles virus

Dolphin morbillivirus

Canine distemper virus

Peste-des-petits-ruminants virus

Phocine distemper virus

Rinderpest virus

Subfamily *Pneumovirinae*

Genus *Pneunovirus*

Human respiratory syncytial virus

Bovine respiratory syncytial virus

Pneunonia virus of mice

Turkey rhinotracheitis virus

Table 1 Members of the *Paramyxoviridae*
Examples from each genus within the *Paramyxoviridae* family. Taken from Lamb and Kolakofsky (1995).

The *Pneumovirinae* are very different from the *Paramyxovirinae* in that, although their gene order is similar, they encode a number of additional genes, (NS1, NS2 and M2), and their attachment protein is different in terms of structure and biological activity.

1.2 Virion Structure

The *Paramyxoviridae*, although pleomorphic in shape, are generally spherical. They consist of a single stranded RNA genome, encapsidated in a helical core structure known as the nucleocapsid. This is surrounded by matrix protein (M) and a host derived lipid bilayer, through which two glycoprotein (HN and F) protrude. A schematic diagram of the *Rubulavirus*, SV5, is given in Fig.1.

The infectious cycle begins with virus atttachment to the target cell surface and fusion with the cell membrane, and is mediated by the glycoproteins. The attachment protein (H, HN or G), interacts with a specific receptor on the host cell surface whereupon the fusion protein (F) fuses with the cell membrane facilitating the release of the nucleocapsid into the cell cytoplasm (Reviewed in Choppin and Compans, 1975).

The nucleocapsid is comprised of the negative sense RNA genome in tight association with the nucleocapsid protein (NP) in a helical core structure which is resistant to nuclease attack. To this, the phosphoprotein (P) and the large protein (L) are attached. It has been reported that V protein is also in association with the nucleocapsid of SV5 (Paterson *et al*, 1995). This nucleocapsid core serves as the template for both the generation of mRNA (transcription) and genomic/anti-genomic RNA (replication) as shown in Fig.2. Transcription and replication are functions of the viral polymerase, which requires both P and L proteins to constitute an active complex (Hamaguchi *et al*, 1983).

Paramyxovirus nucleocapsids are not rigid structures, as they coil and uncoil in response to changes in salt concentration (Heggeness *et al*, 1980). Furthermore, Sendai virus has been shown to exist in several distinct morphological states at normal salt concentration (Engelman *et al*, 1989). The template is copied by the viral polymerase without dissociation of NP from the nucleocapsid core, thus uncoiling of the

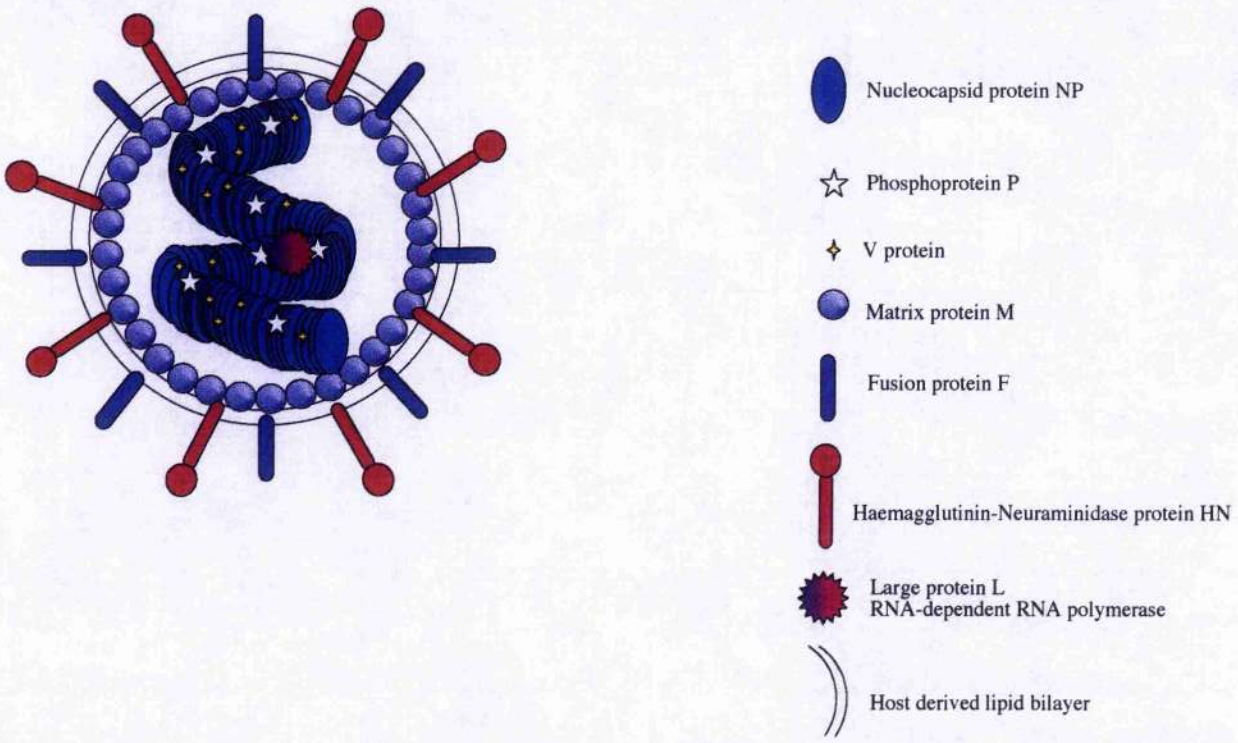


Fig. 1 A schematic diagram of the *Rubulavirus* SV5.

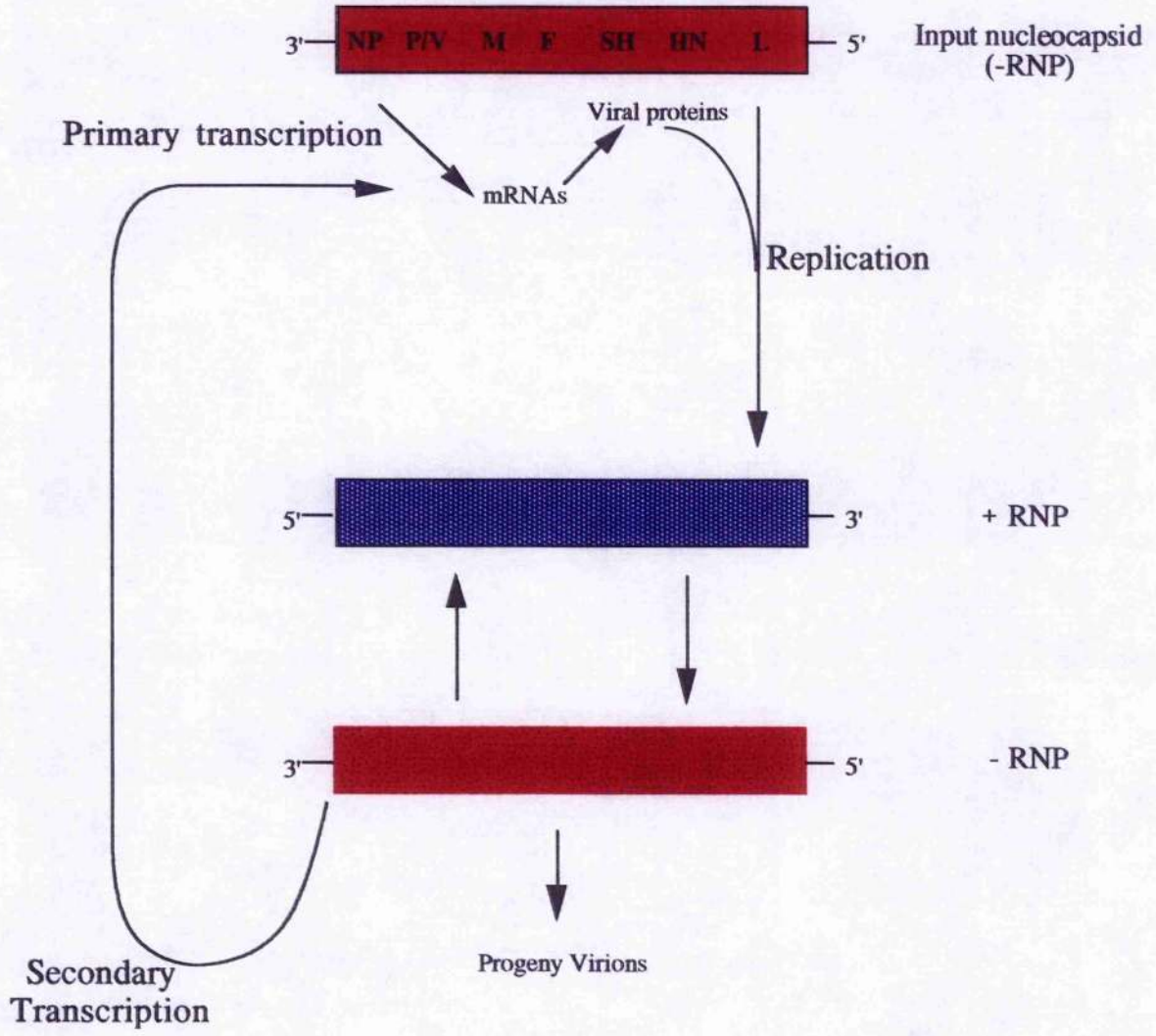


Fig. 2. Overview of transcription and replication steps for the *Paramyxoviridae*

Schematic diagram of the steps involved in transcription and replication of the *Rubulavirus* SV5. The input viral nucleocapsid containing RNA, or ribonucleoprotein (RNP), are indicated as containing negative (-) or positive (+) strand RNA, as discussed in the text. Adapted from Kingsbury (1990).

nucleocapsid core may be necessary for the viral polymerase to gain access to the RNA bases. Therefore, the most extended form of the nucleocapsid structure, where the helix is almost completely unwound, may represent the structure adopted during the copying of the RNA template.

1.3 Genomes and Encoded Proteins

The genomes of the *Paramyxoviridae* are single stranded, non-segmented RNAs of negative polarity, and are approximately 15 kb in length. The genome encodes 6-10 genes (Fig.3) depending on the virus being examined, where a 'gene' refers to a region of genomic RNA which encodes one (or more) specific mRNA(s). Flanking the genes, is a 3' extracistronic sequence known as the leader region and a 5' extracistronic sequence known as the trailer region. Both are around 50 nucleotides in length, are well conserved within the *Paramyxoviridae*, and contain conserved sequences at each end of the genome (Galinski and Weschler, 1991). In human parainfluenza virus type 3 (hPIV3), 33 of the first 39 nucleotides are conserved in the leader and trailer sequences (Galinski, 1988) suggesting these sequences contain signals enabling polymerase attachment to the template and encapsidation of the nascent strand (Blumberg *et al*, 1991). There are sequences found between the genes (i.e. after one gene end sequence and before the next gene start sequence) known as intergenic regions. These are variable in length for the *Rubulaviruses* (1-47 nucleotides) and *Pneumoviruses* (1-56 nucleotides) but are exactly three nucleotides long in both the *Parainfluenzaviruses* and the *Morbilliviruses*. In measles virus (MeV), Sendai virus (SeV) and hPIV3, the intergenic sequences are GAA, GGG and GCT respectively (Galinski and Wechsler, 1991). The influences on viral transcription of these intergenic sequences, and the leader and trailer sequences, are discussed further in section 1.4.2.

1.3.1 The Nucleocapsid Protein (NP)

The nucleocapsid protein (NP, N) is the most abundant protein in the viral nucleocapsid (Lamb *et al*, 1976), and serves several functions in virus transcription/replication. It

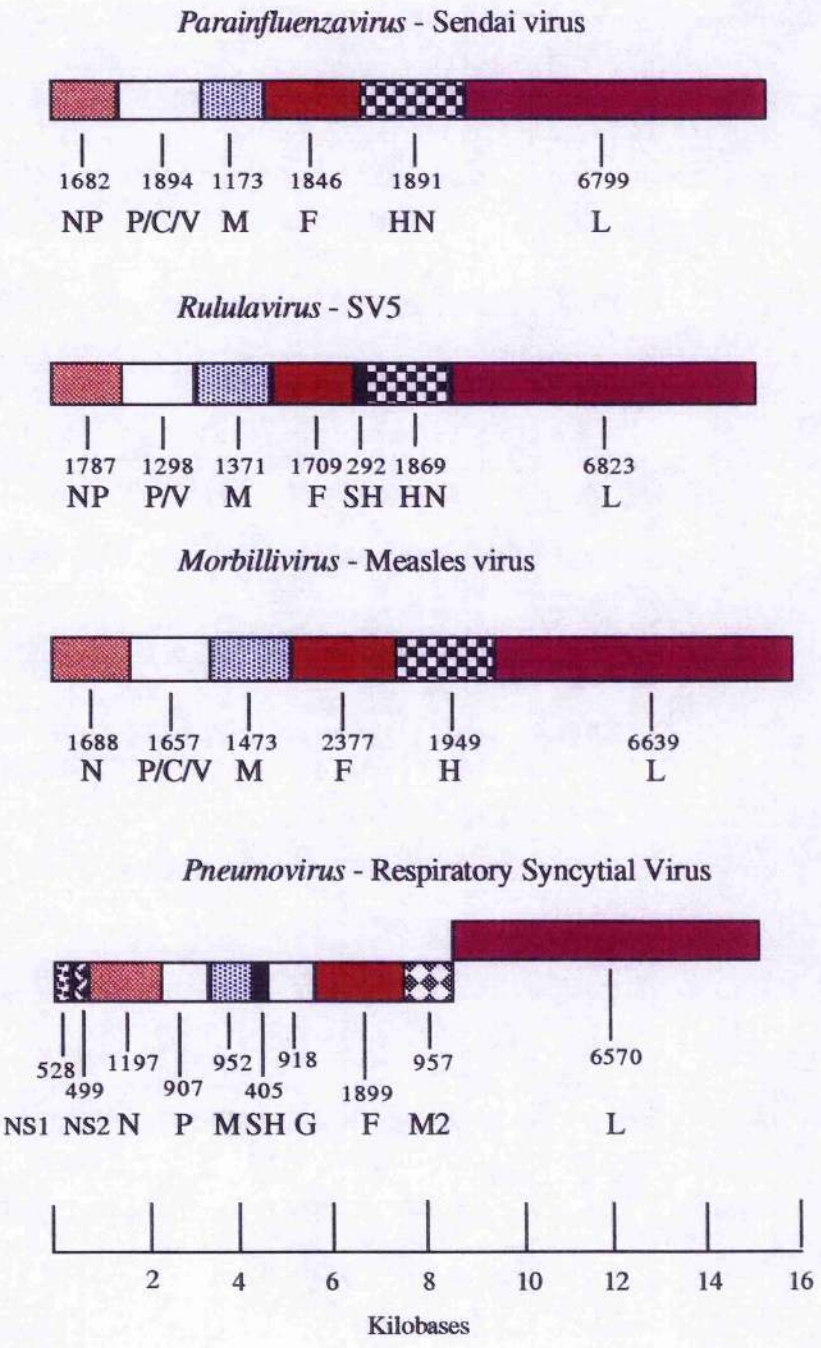


Fig.3 Genome organisation in the *Paramyxoviridae*.

Schematic diagram showing the genome organisation of a representative member from each genus of the *Paramyxoviridae*. The L gene of the *Pneumoviruses* overlaps with that of the M2 gene and is therefore shown as staggared. Adapted from Lamb and Kolakofsky (1995).

firstly encapsidates genomic (and anti-genomic) RNA, giving protection from nuclease attack (Banerjee, 1987), and also gives rise to the characteristic helical structure of the nucleocapsid (Das and Banerjee, 1993; Fooks *et al*, 1993; Buchholtz *et al*, 1993). NP interacts with both the polymerase complex (Kingsbury, 1974) and M protein (Markwell and Fox, 1980), suggesting a role for NP in transcription, replication and virus assembly. Furthermore, it is thought that the intracellular concentration of NP is a major factor in the switch from transcription to replication (Vidal and Kolakofsky, 1989).

The sequences of many NP proteins have been predicted (e.g. Neubert *et al*, 1991; Parks *et al*, 1992; Rozenblatt *et al*,1985; Collins *et al*, 1985) and range from 489-533 amino acids (αα) in length. Protein sequence alignments have suggested that NP contains 2 domains, a large globular N-terminal domain of around 500 amino acids which is relatively conserved between related viruses, and a small, hyper-variable, C-terminal tail region extending from the globular body of the protein (Heggeness, 1981). Trypsin digests of the NDV nucleocapsid (Kingsbury and Darlington, 1968) demonstrated that the C-terminal domain could be cleaved off without affecting the ability of NP to form RNase resistant nucleocapsids. These nucleocapsids were then examined by EM and were found to retain their characteristic helical conformation. This suggested that both the RNA-binding domain and the residues involved in the interactions resulting in helical nucleocapsid formation, were to be found in the globular N-terminal domain. A conserved hydrophobic motif of FX_4YX_4SYAMG (where X is any residue), is found in the middle of the protein, suggesting this may be the site of NP:NP interaction (Morgan *et al*, 1984). However, since this motif contains a number of aromatic amino acid residues (Tyr, Phe or Trp), it has been suggested that this could be the RNA binding site (Morgan *et al*, 1984).

The divergent C-terminus of NP contains many negatively charged residues and the major sites of phosphorylation for the protein. In Sendai virus, a stretch of 7 acidic amino acids (Asp or Glu residues) is thought to be the site of NP:M interaction (Markwell and Fox, 1980).

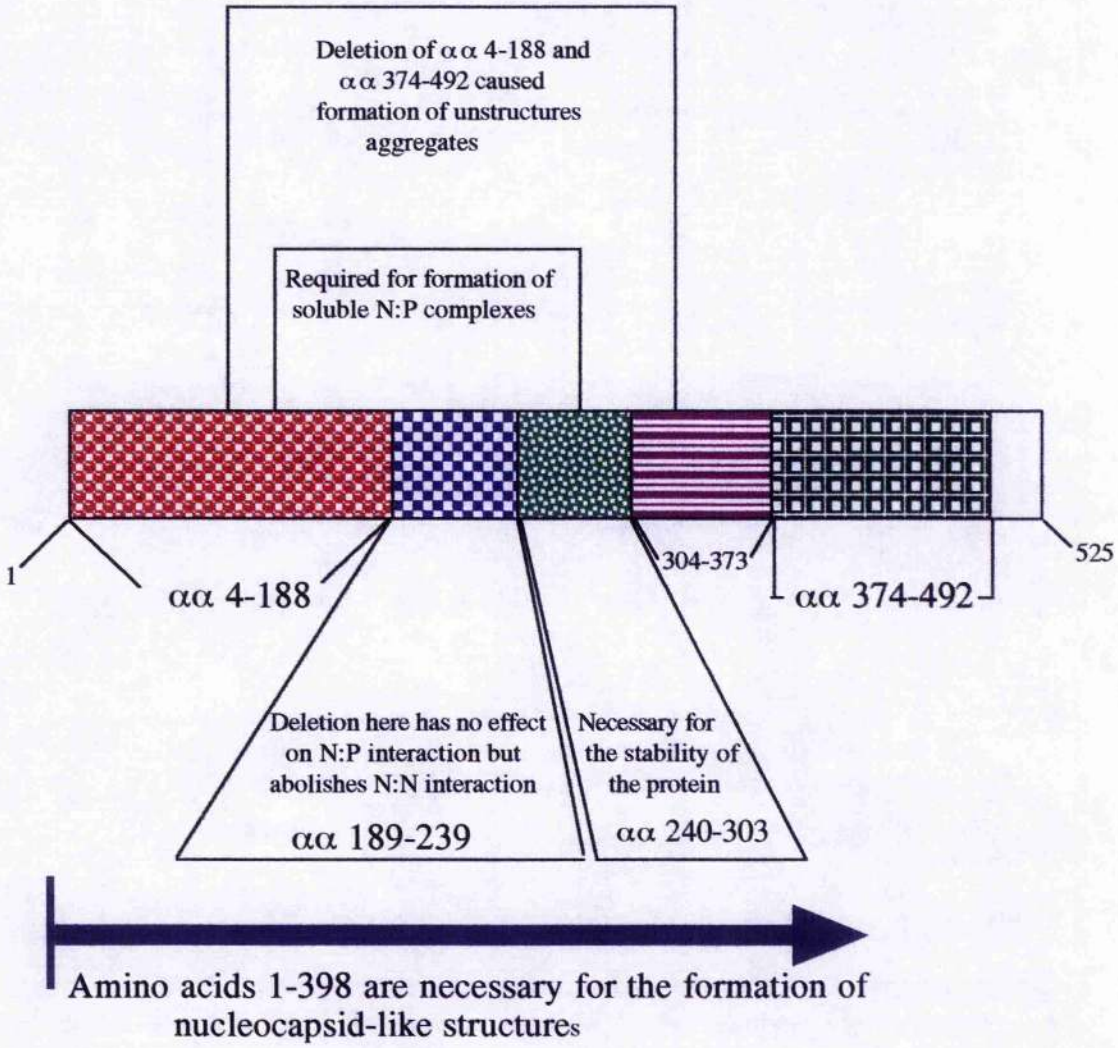


Fig. 4 Schematic diagram of measles virus N protein

Sites of protein:protein interaction on MeV N protein were identified by deletion mutant analysis. Two non-contiguous regions (amino acids 4-188 and 304-373) are required for the formation of soluble N:P complexes, while deletion of amino acids 189-239 did not affect N:P binding. Amino acids 240-303 appear necessary for the stability of the protein. The N-terminal 398 amino acids are all required for the formation of organised nucleocapsid-like particles, since deletion of the central region from amino acids 189-373 completely abolish N:N interaction, and deletion of amino acids 4-188 and 374-492 caused the formation of unstructured aggregates.
Data taken from Bankamp *et al* , 1996.

Structure / function analysis of Sendai virus NP protein found that the C-terminal tail was not essential for encapsidation of a synthetic defective interfering (DI) particle but was required for this template to be functional in subsequent replication assays (Curran *et al*, 1993). The role of the NP C-terminus remains unclear, but since NP protein with C-terminal deletions can form nucleocapsid-like structures containing cellular RNA, it may play a role in the specificity of RNA assembly (Buchholz *et al*, 1993).

During viral replication, soluble (unassembled) NP must be transported to the site of nucleocapsid assembly since genome replication and encapsidation are concurrent (Vidal and Kolakofsky, 1989). There is evidence to suggest that P protein chaperones NP to the polymerase complex (Horikami *et al*, 1992; Buchholz *et al*, 1993; Curran *et al*, 1993) and in doing so, prevents NP from self-aggregating. Evidence presented in this thesis, and elsewhere, suggests that V protein may play a similar role in preventing NP from self-aggregating (Randall and Bermingham, 1996; Horikami *et al*, 1996). A further interaction, probably with the viral polymerase complex, must take place, which allows NP to specifically assemble the nascent RNA and which, presumably also releases P.

Deletion mutant analysis on both Sendai virus and measles virus, have identified areas of NP:NP and NP:P interaction (Buchholz *et al*, 1993, Bankamp *et al*, 1996) as shown in Fig.4. The N-terminal 398 amino acids were required for the formation of nucleocapsid-like structures and deletion of amino acids 189-373, abolished NP:NP interaction. Two regions (amino acids 4-188 and 304-373) were also identified as being required for soluble N:P complex formation. Measles N also undergoes a conformational maturation upon phosphorylation, whereupon it is available for assembly into nucleocapsid structures (Gombart *et al*, 1993).

1.3.2 Proteins encoded by the P Gene

When comparing the P genes, a major difference is found between the *Pneumovirinae* and the *Paramyxovirinae*. The P gene of the *Pneumovirinae* gives rise to a single P protein while that of the *Paramyxovirinae*, encodes multiple proteins by the use of

multiple reading frames. These reading frames are accessed during viral transcription either by the use of a variable number of initiation codons or by a process known as RNA editing. For the latter, a frameshift is generated by the addition of non-templated residues due to a polymerase stuttering mechanism. Thus, proteins with common N-terminal sequences but unique C-termini are generated. These proteins, and their generation, are discussed in more detail below.

1.3.2.1 P Protein

The sequences of many paramyxovirus P proteins have now been predicted and are found to be variable in length within the family (e.g. Bellini *et al*, 1985, Thomas *et al*, 1988, Shioda *et al*, 1986, Southern *et al*, 1990, Spriggs and Collins, 1986). The P proteins, named because they are highly phosphorylated, play a pivotal role in RNA synthesis. P functions as a subunit of the viral polymerase as both P and L proteins are necessary to constitute an active polymerase complex (Hamaguchi *et al*, 1983). It also binds soluble (unassembled) NP, forming an NP-P complex which prevents NP from self aggregating and possibly delivers NP to the nascent RNA chain for encapsidation during viral replication (Curran *et al*, 1995a). As previously mentioned, there is some evidence to suggest that NP-P complexes prevent NP from non-specifically encapsidating cellular RNA (Curran *et al*, 1993, Buchholz *et al*, 1993, Masters and Banerjee, 1988).

The P protein can be divided into an N-terminal domain, a C-terminal domain and a hypervariable region in between as shown in Fig.5. The N-terminal region of P proteins contain the phosphorylation sites (Das *et al*, 1995; Byrappa *et al*, 1995;Vidal *et al*, 1988) and areas involved in RNA synthesis (Curran *et al*, 1994 and 1995a).

The immediate N-terminal residues (amino acids 1-77) in Sendai virus (SeV) were shown to be essential for RNA encapsidation (Curran *et al*, 1994). This study also demonstrated that 9 amino acids within this region (amino acids 33-41) were required to bind soluble NP for delivery to the nascent chain during genome replication, therefore acting as a chaperone for NP.

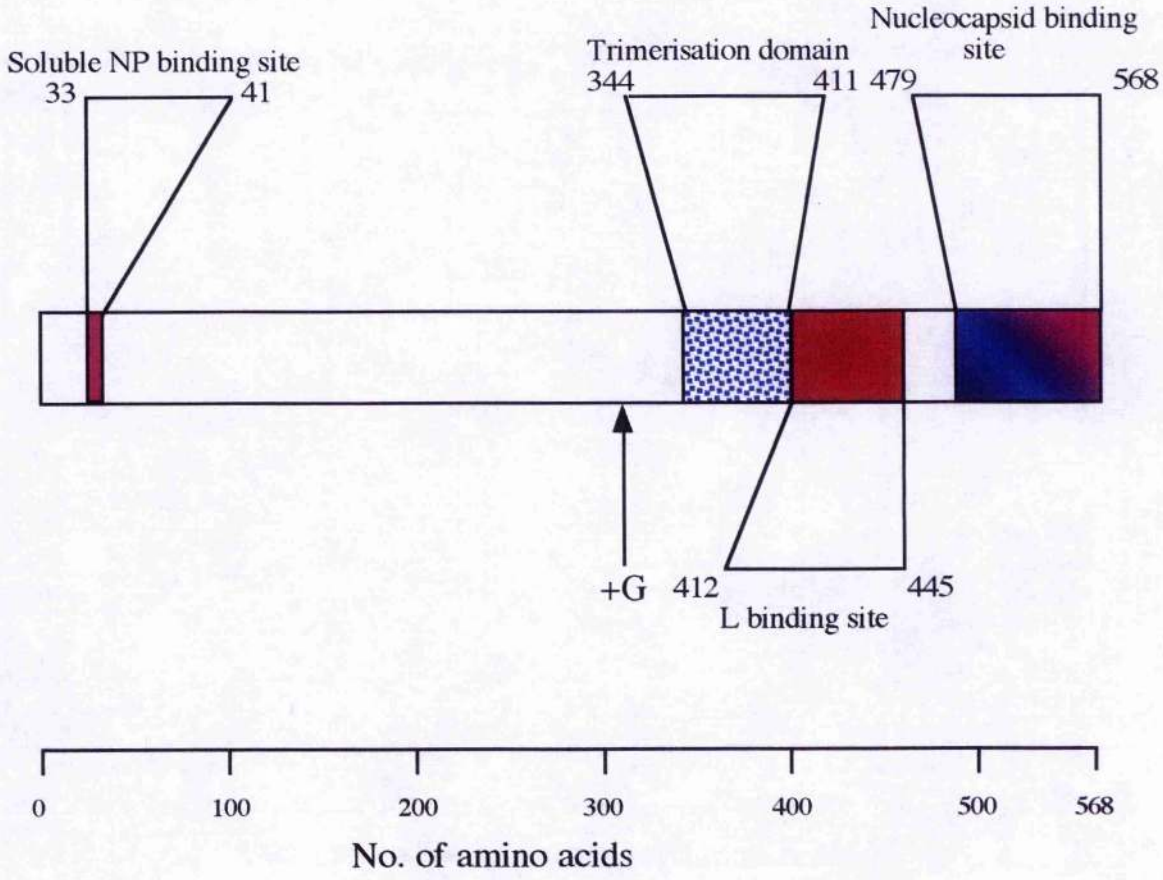


Fig.5 Schematic diagram of SeV P protein

Illustration of the areas of contact beween P and the other viral proteins indicated. Residues 33-41 are required for the chaperoning of unassemled NP during the nascent chain assembly step of genome replication. Two blocks within the C-terminal 40% of the protein (αα344-411 and 479–568) are involved in binding to nucleocapsids. Residues 344-411 are also required for trimerisation and only trimers bind to the nucleocapsid template. Residues 412-478 represent the stable binding site for the L protein. The site for the addition of a non-templated G residue at codon 308 during mRNA synthesis is also indicated. Adapted from Curran (1996)

The C-terminal portion of SeV P protein contains 2 nucleocapsid (assembled NP) binding sites at amino acids 345-411 and 479-568 (Ryan and Kingsbury, 1988; Ryan *et al*, 1991), one of which (amino acids 344-411) is also the region essential for oligomerisation of the P protein (amino acids 344-411) into homotrimers (Curran *et al*, 1995) and only trimers bind to the nucleocapsid template. Between the NP binding sites, lies the L binding site (amino acids 412-445) which loops out to contact L and does not overlap with either of the NP binding sites (Curran *et al*, 1994; Smallwood *et al*, 1994).

1.3.2.2 C Proteins

Only members of the *Parainfluenzavirus* and *Morbillivirus* genera express small basic C proteins from their P genes (Barrett *et al*, 1985, Bellini *et al*, 1985, Spriggs and Collins, 1986). SeV encodes 4 'C-like' proteins, namely C', C, Y1 and Y2 (Curran and Kolakofsky,1989) while hPIV1 expresses C', C and Y1 (Power *et atl* 1992). These proteins are expressed both *in vivo* and *in vitro* and are generated from a variable number of start codons, including non-AUG initiation codons (reviewed in Kolakofsky *et al*, 1991). SeV C' and C (But not Y1 or Y2) proteins have been shown to inhibit mRNA synthesis and genome replication by possibly forming inactive complexes with L protein. It has been suggested that the C proteins exhibit a negative regulatory role during virus infection to limit both genome amplification and mRNA synthesis (Cadd *et al*, 1996; Curran *et al*, 1992). Given the very different levels of C expression among the different *Paramyxoviridae*, the viruses may have differing requirements for down-regulation of genome amplification and expression in the later stages of infection. A C-deleted measles virus has recently been found to be viable and essentially without mutant phenotype in cell culture (Radecke and Billeter, 1996) as has the analogous rhabdovirus, vesicular stomatitis virus (VSV; Kretzschmar *et al*, 1996). However, whether these C-deleted viruses will exhibit any phenotypic effect *in vivo*, remains to be determined.

1.3.2.3 V, W and I Proteins

The P genes of most of the *Paramyxovirinae* are able to express two mRNA species by a mechanism of RNA editing which inserts non-templated G residues. It is a virus specific event which takes place co-transcriptionally by reiterative copying of a short C-stretch at a specific region on the genome template (Vidal *et al*, 1990a). The septanucleotide consensus motif (3'- UUU/CUCCC -5'), (Fig. 6) including the C-stretch, has been proposed as a requirement for editing (Park and Krystal, 1992). A 'stuttering' model proposes that the polymerase pauses at this site, and when the pause is sufficiently long, slippage of the nascent mRNA occurs by one or two nucleotides, thereby reiteratively inserting one or two Gs (Vidal *et al*, 1990b).

For the *parainfluenzaviuses* (SeV, hPIV3) and *Morbilliviruses* (measles virus), the unedited mRNA that is a faithful copy of the genome, encodes the P protein while the edited mRNA, with 1G addition, encodes the V protein (Vidal *et al*, 1990a; Galinski *et al*, 1992). However, for the *Rubulaviruses* (SV5, MuV), the unedited mRNA encodes the V protein while the edited mRNA, with a 2G insertion, encodes the P protein (Thomas *et al*, 1988; Southern *et al*, 1990; Paterson and Lamb, 1990; Ohgimoto *et al*, 1990). In either case, the P and V proteins are amino-coterminal, while the -1 or -2 frame is used to generate unique C-termini from the edited mRNA.

For the *Morbilliviruses* and some of the SeV group which require a 1G insertion to access the V open reading frame (ORF), a 2G insertional event (at a much reduced frequency) leads to a protein called W. This is essentially the N-terminal part of the P protein up to the editing site as the downstream ORF is quickly closed by a stop codon (Vidal *et al*, 1990a,b; Kolakofsky *et al*, 1991). The remaining P gene product of the *Rubulaviruses*, represents the N-terminal part of the V or P protein up to the editing site, referred to as the I protein, is accessed by the insertion of 4Gs rather than 2Gs (Paterson and Lamb, 1990).

Of the distinct P gene products, the V protein is of particular interest because of its almost universal conservation in all three genera of the *Paramyxovirinae*. To date, human parainfluenza virus type 1 (hPIV1) seems to be the only exception, as it has neither the consensus editing motif nor the V ORF (Matsuoka *et al*, 1991; Power *et al*,

MeV	A A U **U U** U **U C C C** G U g U C	5'
SeV	g u U **U U** U **U C C C** G U a U C	5'
MuV	A A A **U U** C **U C C C** C c c g g	5'
SV5	A A A **U U** C **U C C C** C g u c C	5'
consensus	U U Y U C C C	

Fig. 6 Consensus Sequence for P mRNA Editing

The four paramyxovirus minus-strand genome sequences where G insertion are known to occur, written 3' to 5' are shown. Exact homologies are highlighted in underlined bold script and other homologies are given in capital letters. The consensus sequence is given underneath.
Taken from Vidal *et al* (1990)

1992; Rochat *et al*, 1992). The V protein is structurally characterised by a highly conserved cysteine-rich domain in its carboxy-terminal half, which is fused to the amino-terminal half of the P protein. The V proteins of MeV, SV5 and NDV have all been shown to bind zinc (Liston and Briedis, 1994; Paterson *et al*, 1995; Steward *et al*, 1995), although the functional significance of this remains unclear.

The V proteins of SeV and measles virus (MeV) are not present in virions and are not associated with the RNP, probably because these V proteins are missing the trimerisation site, L binding site and assembled NP (nucleocapsid) binding sites, which are located in the C-terminal half of the protein (see 1.3.2.1 for further details). However, the V protein of the *Rubulaviruses* does appear to be present in virions (Paterson *et al*, 1995; Takeuchi *et al*, 1990, Kawano *et al*, 1993) and unlike the *Parainfluenza*- and *Morbilliviruses*, their N-terminal halves are predicted to be basic rather than acidic in character. The strong conservation of the V ORF throughout almost all the *Paramyxovirinae* suggests that it may interact with a structurally invariant surface e.g. a cellular protein which is highly conserved from chickens (NDV) to man. Interactions between V and cellular proteins have been reported for MeV (Liston *et al*, 1995) and SV5 (Precious *et al*, 1995) although the cellular proteins involved were of markedly differing size.

The function of V protein has been studied using SeV defective interfering (DI) particles as a genome analogue. The V (and W) protein was found to inhibit DI genome replication (Curran *et al*, 1991a) possibly by interferring with the RNA encapsidation step (Curran *et al*, 1994) but did not appear to affect viral mRNA synthesis (Curran *et al*, 1994). However, when a V-minus (but W-augmented) SeV was engineered from cDNA, it was found to replicate normally in cell culture and embryonated chicken eggs (Delenda *et al*, 1997). Moreover, when a similar V-minus SeV was generated which also did not produce W protein, it displayed markedly increased gene expression and cytopathogenicity at the cellular level *in vitro*, but was strongly attenuated in pathogenicity for mice. Therefore, V may act in animal infections to avoid the anti-viral response of the host (Kato *et al*, 1997a).

The recovery of V-minus viruses from cDNA indicates that V protein is not an essential factor for SeV replication. This suggests that V may be an accessory protein, not absolutely required by all viruses, and therefore some viruses have adapted to do without them e.g. hPIV1. Similar conclusions have been drawn for the role of C protein in MeV and VSV (Radecke and Billeter, 1996; Kretzschmar *et al*, 1996; and section 1.3.2.2).

1.3.3 The Large (L) Protein

The large (L) protein is, as its name suggests, the largest and least abundant protein in the virion. Both P and L proteins are necessary to constitute an active polymerase complex (Hamaguchi *et al*, 1983), although L is thought to be responsible for the majority of enzymatic activities involved in viral transcription and replication. Many of these activities have been demonstrated in genetic or biochemical studies on the L protein of the rhabdovirus VSV. Viral mRNA capping, methyl-transferase activities and polyadenylation have all been attributed to VSV L protein (Abraham *et al*, 1975; Hercyk *et al*, 1988; Hunt *et al*, 1984). The L proteins of VSV and SeV also have kinase activity since they phosphorylate both NP and P proteins (Sanchez *et al*, 1985; Einberger *et al*, 1990).

Sequence alignments with several L proteins from rhabdoviruses and paramyxoviruses have shown six conserved regions, separated by variable regions, suggesting a structure of concatenated functional domains (Poch *et al*. 1990). Furthermore, four short conserved sequence motifs were found in domain III of the L proteins which were also conserved in the RNA-dependent RNA polymerases of other virus families (Poch *et al*, 1990). These motifs surrounded an invariant pentapeptide (QGDNQ) and were suggested to represent important elements of the active site for template recognition and/or phosphodiester bond formation by the L proteins.

The N-terminal of both SV5 and MeV L proteins have been shown to contain sequences important for stable L-P complex formation (Parks, 1994; Horikami *et al*, 1994). Site directed mutagenesis in conserved domain I (amino acids 348-379) of SeV

L protein, showed that this region was important for both transcription and replication functions of the protein (Chandrika *et al*, 1995).

1.3.4 Matrix (M) Protein

The matrix (M) protein is the most abundant protein in the virion and lies between the nucleocapsid and the glycoprotein-containing envelope. The sequences of many of the *Paramyxoviridae* M proteins have been predicted (Bellini *et al*, 1986; Blumberg *et al*, 1984; Satake and Venkatesan, 1984; Sheshberaderan and Lamb, 1990), indicating they are basic proteins of between 341-375 amino acid residues. M protein is hydrophobic in nature but does not have any hydrophobic stretches long enough to span a lipid bilayer (Reviewed in Peeples, 1991). Very little is known about the structure of the M protein, but a recent report on the M protein of the rhabdovirus VSV suggested a rod-like shape (Barge *et al*, 1996).

It is generally accepted that M protein is the central organiser of virion assembly, as its hydrophobic character allows it to interact with the glycoprotein-containing plasma membrane and its positively charged residues allows interaction with the newly synthesized (and negatively charged) nucleocapsids (Yoshida *et al*, 1976; Sheshberaderan and Lamb, 1990; Morrison, 1988). M protein can also bind to itself and forms a paracrystalline array with defined periodicity on the inner side of the infected cell plasma membrane (Bachi, 1980). This self-association and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (Peeples, 1991). It has also been reported that M protein binds actin (Giuffre *et al*, 1992; Bohn *et al*, 1986) which may also prove important for virus budding.

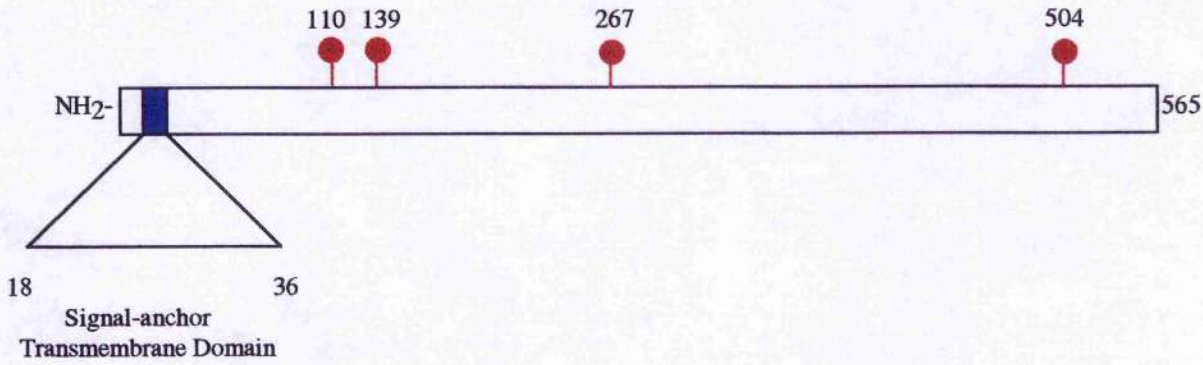
There is clear evidence that M proteins of VSV and influenza virus down-regulate transcription from RNP cores (Ye *et al*, 1989; Zvinarjev and Ghendon, 1980; Ye and Wagner, 1992; Black *et al*, 1993) and there is some evidence to suggest measles virus M protein is an endogenous inhibitor of RNP transcription (Suryanarayana *et al*, 1994). This study, and elsewhere (Cattaneo *et al*, 1988a; Cattaneo *et al*, 1988b) suggested that MeV encephalitis arises from selective hypermutation in the M gene resulting in either

an inability of the M protein to bind RNPs or associate with budding structures. Therefore, the striking abundance of RNP cores in brain cells of patients with the fatal persistent measles virus infection, subacute sclerosing panencephalitis (SSPE), could possibly result from unrestricted transcription and replication of RNP cores as a result of certain mutated M proteins. These mutated M proteins could either be defective in transcription inhibition, or may just have lost their ability to bind RNP cores (Suryanarayana *et al*, 1994).

1.3.5 The Envelope Glycoproteins

The membranes of *Paramyxoviridae* virions are studded with spike structures which can readily be visualised by electron microscopy. Biochemical characterisation of these structures has revealed there to be two different glycoprotein spikes on the surface of the virion. One acts as an attachment protein which serves to bind the virus to the cell suface and is composed of a protein termed haemagglutinin-neuraminidase (HN) protein in the *Parainfluenza-* and *Rubulavirus* genera, Haemagglutinin (H) protein in the *Morbillivirus* genus and G protein in the *Pneumovirus* genus. As their names suggest, the attachment protein of the *Parainfluenza-* and *Rubulavirus* genera also contains a neuraminidase activity not demonstrated for the attachment proteins of the other genera (Scheid *et al*, 1972). The other spike is composed of the fusion protein and mediates the fusion of the viral membrane with that of the host cell, facilitating the release of the nucleocapsid into the cell cytoplasm. This protein also mediates cell-to-cell spread of the genetic information, and a prominant feature of the cytopathic effect of paramyxoviruses is syncytium formation (reviewed in Choppin and Compans, 1975). The virus utilises the host cell pathways for synthesis, transport and post-translational modifications of the surface glycoproteins for insertion into the plasma membrane. Once the two glycoproteins are inserted into the plasma membrane of infected cells, they are subsequently incorporated into virions as the virus buds from the cell surface.

A. Haemagglutinin-Neuraminidase of SV5



B. Fusion Protein of SV5

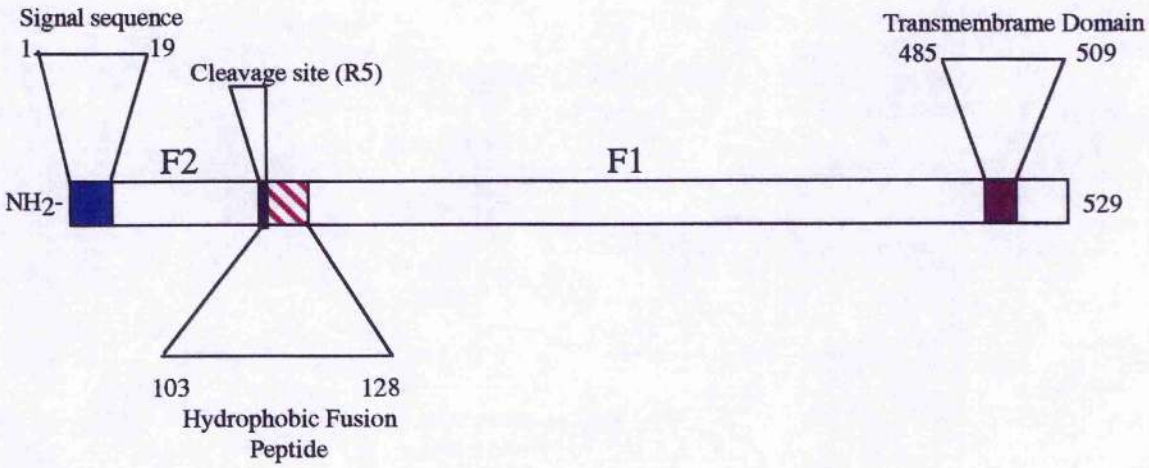


Fig. 7 Schematic diagram of the paramyxovirus glycoproteins

(A) Haemagglutinin-neuraminidase attachment protein of SV5 indicating the signal anchor transmembrane domain and the sites used for the addition of N-linked carbohydrates.
(B) Fusion protein of SV5 indicating the position of the signal sequence, the transmembrane domain, the cleavage site and the hydrophobic fusion peptide.
Adapted from Lamb and Kolakofsky (1995)..

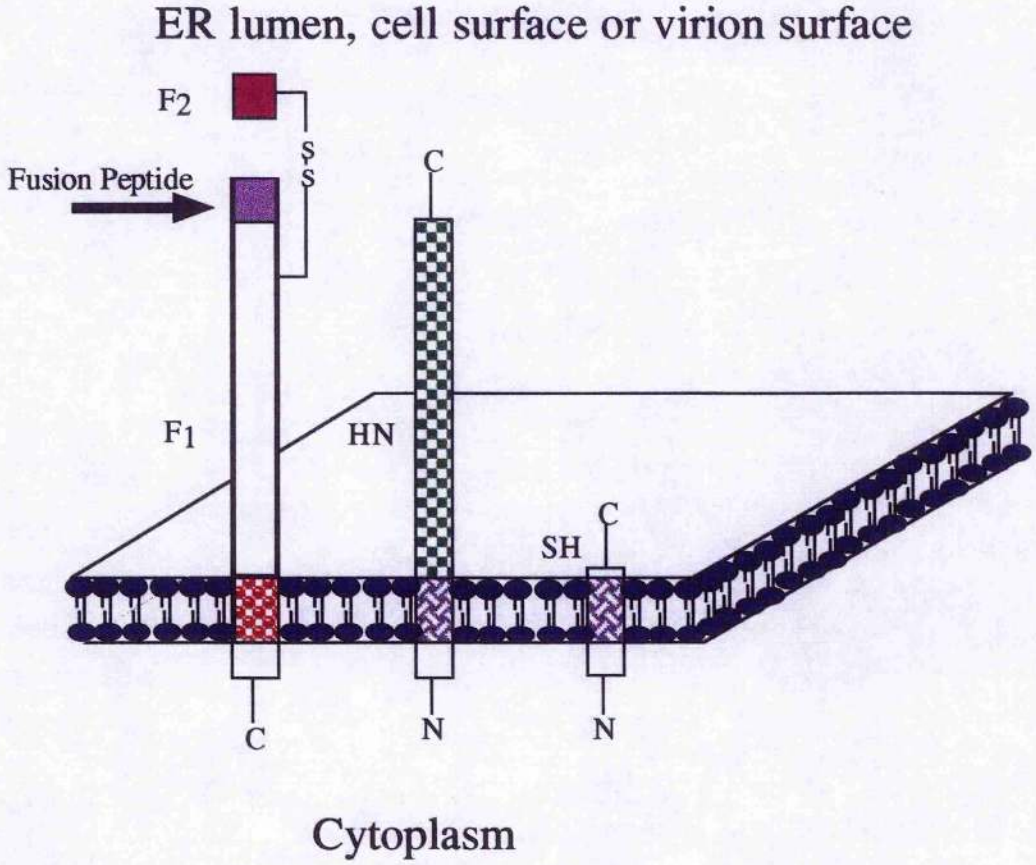


Fig.8 Schematic diagram of integral membrane proteins
The type I and type II integral membrane proteins F and HN of the *Paramyxoviridae*. Of the *Paramyxovirinae*, only SV5 has been shown to encode an SH protein, although there is an SH ORF in mumps virus.
Adapted from Lamb and Kolakofsky, 1995.

1.3.5.1 Attachment Protein

The HN protein of the *Parainfluenzaviruses* and the *Rubulaviruses* is a multifunctional protein. It is responsible for the binding of the virus to sialic acid-containing cellular receptors such as glycoproteins or glycolipids, and mediates enzymatic cleavage of sialic acid from the surface of virions and the surface of infected cells (Morrison and Portner, 1991). By analogy to the influenza virus neuraminidase, it seems likely that the neuraminidase activity of HN prevents self-aggregation of the virus particles when budding from the plasma membrane.

The sequences of many attachment proteins have been predicted (Blumberg *et al*, 1985a; Hiebert *et al*, 1985a; Alkhatib and Briedis, 1986; Wertz *et al*, 1985) indicating that they are 565-582 amino acids in length. HN proteins are type II integral membrane proteins, which span the membrane once, and contain a single hydrophobic-domain at the N-terminus. This acts as both a signal and anchorage domain, leaving a long C-terminal ectodomain (Reviewed in Morrison, 1988). Conserved cysteine, proline and glycine residues between paramyxovirus HN proteins, has suggested a similarity in protein structure across the family (Figs. 7A and 8).

HN exists as a disulphide linked dimer which forms a non-covalently linked tetramer (Ng *et al*, 1989). The stable formation of the tetramer is dependent on the transmembrane domain and the cytoplasmic tail of the protein (Ng *et al*, 1989; Parks and Lamb, 1990; McGinnes *et al*, 1993). The intramolecular disulphide bonds are formed during folding of the protein (McGinnes and Morrison, 1994a) forming the covalently linked dimer (McGinnes and Morrison, 1994b). This disulphide bond formation has recently been shown to be cotranslational and important to the subsequent folding of a conformationally normal and active HN protein (McGinnes and Morrison, 1996). Furthermore, during its synthesis, HN binds the cellular protein GRP78-BiP (Ng *et al*, 1989) which prevents transport of defective proteins from the endoplasmic reticulum to the Golgi network (Pelham, 1986; Hurtley *et al*, 1989).

HN has 4-6 sites for the addition of N-linked carbohydrate chains (Ng *et al*, 1990) which, in the case of MuV and SeV, is necessary for incorporation into virions (Herrler and Compans, 1983; Nakamura *et al*, 1982). However, this is not the case for NDV,

where glycosylation is required for virus infectivity but not incorporation into virions (Morrison *et al*, 1981). These glycosylation sites are found towards the C-terminal end of HN (Morrison, 1988), suggesting the protein consists of a globular head, containing the enzymatic activites, supported by a stalk-like structure which is inserted into the plasma membrane (Thompson *et al*, 1988). In addition, HN contains a conserved sequence, NRKSKS, which is similar to the known sialic acid binding site of influenza virus neuraminidase (Morrison and Portner, 1991). On comparing the primary protein sequences of the paramyxovirus HN proteins with influenza virus NA, four regions containing conserved motifs were found. These motifs in NA, and by analogy in HN, were postulated to be brought together to form the neuraminidase active site in the conformationally mature protein (Colman *et al*, 1993). Supporting this hypothesis was the finding that measles virus H, which does not have neuraminidase activity, contains only one of these conserved motifs.

There is still some disagreement whether the sites of haemagglutinin and neuraminidase activities are combined (Scheid and Choppin, 1974) or are separate. If the analogy with influenza virus NA can be continued to include an NA with haemagglutinating activity, then the HN activities would be expected to be located at separate sites (Laver *et al*, 1984).

As mentioned previously, the H protein of the *Morbilliviruses* had no neuraminidase activity (Morrison and Portner, 1991 and references therein). It specifically interacts with the CD46 cellular receptor molecule (Dorig *et al*, 1993; Naniche, 1993). Since CD46 is expressed at a low level on the cell surface, aggregation during virus budding is unlikely, thus negating the need for *Morbilliviruses* to exibit neuraminidase activity to free themselves from the cell surface.

Like HN, measles virus H is a type II integral membrane protein and also forms disulphide linked dimers which are also possibly homotetramers. They also contain N-linked carbohydrate chains which are located in the N-terminus of the protein (Alkhatib and Briedis, 1986), unlike HN where the glycosylation sites are distributed towards the C-terminus. These H glycosylation sites are collectively required for folding of the protein into a native structure (Hu *et al*, 1994).

The G protein of the *Pneumovirinae* is, like HN and H, a type II integral membrane protein but has neither haemagglutinin nor neuraminidase activities. At 298 amino acids, it is much smaller than either HN or H (Wertz *et al*, 1985) and is found in infected cells in both a proteolytically cleaved, soluble form, and membrane bound. The protein is extensively modified by the addition of both O-linked and N-linked oligosaccharides (Wertz *et al*, 1985; Grober and Levine, 1985). These differences have led to speculation that G protein has a different evolutionary ancestry to HN and H, and is more structurally similar to a group of cellular mucinous proteins (Wertz *et al*, 1985; Sullender and Wertz, 1991).

1.3.5.2 Fusion (F) Protein

The fusion (F) protein of the *Paramyxoviridae* mediates fusion of virus to the host cell membrane and between the infected cell to the adjacent cell, promoting viral spread (Choppin and Compans, 1975). F is synthesised as an inactive precursor F_0, and subsequently cleaved to F_1 and F_2 subunits, which are disulphide linked in the biologically active form (Scheid and Choppin, 1974, and 1977). The protein sequences of the F proteins have been predicted (Blumberg *et al*, 1985b; Paterson *et al*, 1984a; Richarson *et al*, 1986; Elango *et al*, 1985) indicating the encoded proteins contained 540-580 amino acid residues (Figs. 7B and 8).

F is a type I integral membrane protein that spans the membrane once and contains a cleavable signal sequence at the N-terminus of the protein. It has a typical three-domain structure, consisting of a large, relatively hydrophilic domain external to the virion, a second domain of 20 or more uncharged residues that anchors the protein to the lipid bilayer, and immediately adjacent, a hydrophilic C-terminal domain, which exists on the inner side of the virion bilayer or host cell plasma membrane (Morrison and Portner, 1991). Although no overall homology between the different paramyxovirus F protein sequences was found, similar placement of cysteine, proline and glycine residues, together with the hydrophobicity of the proteins, suggested a similar structure for the F proteins.

The *Parainfluenzaviruses* and the *Rubulaviruses* F_1 and F_2 subunits are glycosylated by the addition of N-linked carbohydrate chains (Morrison and Portner, 1991). In the *Morbilliviruses*, F_2 is glycosylated while F_1 is not (Alkhatib *et al*, 1994). The cleavage of F_0 to the active molecule is an important determinant of virus host range and pathogenicity (Scheid and Choppin, 1974; Nagai *et al*, 1976). This proteolytic cleavage in the Golgi network, results in a new C-terminus of F_1 and a conformational change in the molecule, exposing a previously hidden hydrophobic region (Hsu *et al*, 1981; Kohama *et al*, 1981). This region has been identified as the first 20 N-terminal residues of F_1, and is known as the fusion peptide. The paramyxovirus fusion peptides are thought to intercalate into target membranes, initiating the fusion process (Novick and Hoekstra, 1988) and have been shown to act as a transmembrane anchor domain, converting former soluble proteins to membrane bound forms (Paterson and Lamb, 1987).

F is the major glycoprotein involved in virus-cell and cell-cell fusion, but some disagreement remains over the ability of F alone to promote fusion. When cDNAs encoding the SV5 or measles virus F are expressed, syncytium formation is observed (Alkhatib *et al*, 1990 and 1994; Horvath *et al*, 1992). However, for many more of the *Paramyxovirinae*, co-expression of F and HN is necessary for syncytium formation (Cattaneo and Rose, 1993; Ebata *et al*, 1991; Tanabayashi *et al*, 1992). Furthermore, the requirement for the HN protein is generally restricted to HN proteins from the same species of virus (Cattaneo and Rose, 1993; Wild *et al*, 1994; Heminway *et al*, 1995). It had also been proposed that the HN protein may trigger the conformational change in F which may allow the fusion peptide to insert into the membrane (Lamb, 1993). For this interaction between F and HN to occur, the fusion promoting domain of HN (amino acids 59-140 in SeV), may play a central role in a virus type-specific manner (Tanabayashi and Compans, 1996).

1.3.5.3 Other Envelope Glycoproteins

The *Rubulavirus* SV5 expresses a small hydrophobic protein, designated SH, from an ORF situated beween those of F and HN (Fig.3). SH protein is 44 amino acid residues

in length (Hiebert *et al*, 1985b) and is expressed on the infected cell surface (Hiebert *et al*, 1988). This study also demonstrated SH to be a type-II integral membrane protein as the N -terminal domain was expressed on the cytoplasmic face of the plasma membrane, and the C-terminus, of approximately 5 amino acid residues, was expressed on the cell surface (Fig.8). The function of SH in the infectious life cycle of the virus has not yet been determined.

For mumps virus, a similar ORF had been identified and indeed an mRNA transcript derived from this ORF has been detected (Elango *et al*, 1989; Elliot *et al*, 1989). However, attempts to detect SH protein have been unsuccessful. From the mRNA sequence, the predicted SH protein from mumps virus does contain a hydrophobic domain sufficient to span a lipid bilayer.

The *Pneumovirus* RSV also encodes an SH protein whose ORF lies between M and G on the genome (Fig.3), (Collins *et al*, 1986; Olmstead and Collins, 1989). It contains 64 amino acid residues and is also a type II integral membrane protein with no known function in the virus life cycle.

1.4 Genome Replication

A schematic diagram giving an overview of the paramyxovirus lifecycle is shown in Fig. 9. This depicts all aspects of viral transcription and replication taking place in the cell cytoplasm. In summary, the virus first contacts the host cell surface via attachment protein interaction with specific receptors. This interaction facilitates virus fusion with the cell membrane, releasing the nucleocapsid into the cytoplasm. The nucleocapsid acts first as a template for viral transcription, thus generating viral proteins, and then as a template for replication, to generate further nucleocapsids for assembly and release as progeny virions. These processes are described in detail below.

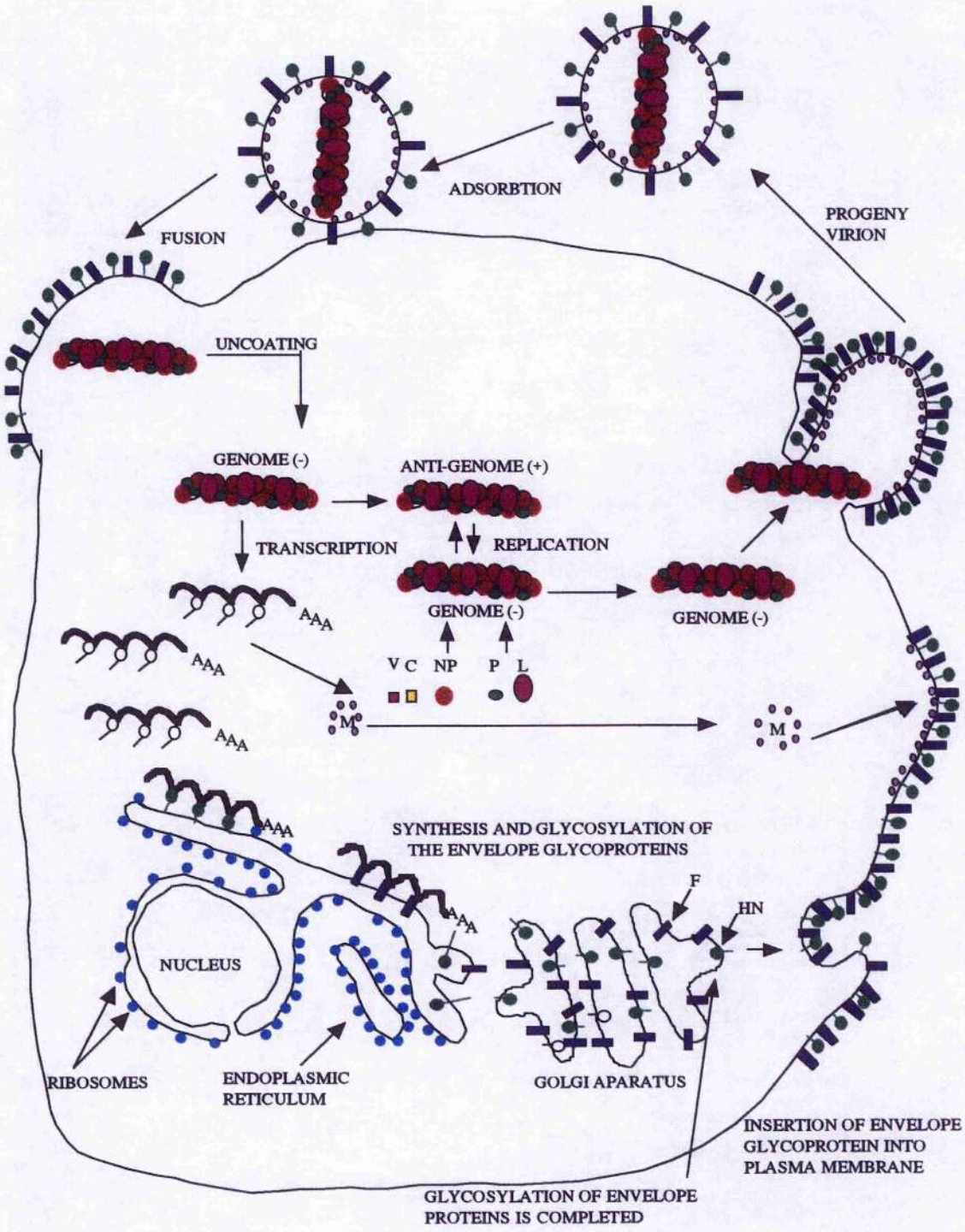


Fig. 9 Schematic diagram of the paramyxovirus life cycle. Adapted from Lamb and Kolakofsky (1995).

1.4.1 Virus Adsorption and Entry

The attachment protein of the *Paramyxoviridae* mediates the first stage of virus adsorption to the host cell surface. As mentioned previously, the specific receptors involved in attachment by the *Parainfluenzaviruses* and the *Rubulaviruses* have long been accepted as sialic acid containing molecules such as glycoproteins and gangliosides (Markwell *et al*, 1981). The *Morbilliviruses*, however, have a different receptor which was identified as the cell surface protein CD46 (Dorig *et al*, 1993; Naniche *et al*, 1993) while the receptor for the *Pneumoviruses* is not yet known.
Once the virus had adsorbed to the cell surface receptor, the virus fuses with the cell membrane. The fusion mechanism in some viruses needs both the HN and the F proteins as discussed earlier (1.3.4.1 and 1.3.4.2). The attachment protein (H or HN) has been proposed to trigger a conformational change in the fusion (F) protein. This conformational change involves the insertion of the fusion peptide into the host cell lipid bilayer resulting in fusion with the host cell membrane and subsequent release of the nucleocapsid into the cell cytoplasm.
Once inside the cell, the nucleocapsid must be uncoated in preparation for transcription. In the virus particle, M protein is thought to act as a bridge between the nucleocapsid and the virus envelope, and is also known to be responsible for inhibiting virus transcription during virus assembly (see 1.3.3 for details). Therefore there must be some mechanism to remove M from newly released nucleocapsids before transcription commences which is not yet understood.

1.4.2 Viral Transcription

Most of the information on RNA transcription by the non-segmented RNA viruses is based on studies on the rhabdovirus vesicular stomatitis virus (VSV) or SeV (Emerson, 1982; Rose, 1980; Vidal and Kolakofsky, 1989). Evidence from these studies support a model in which the polymerase complex enters the genome at a promoter in the 3' extracistronic leader region. Transcription proceeds along the linear array of genes in a

sequential termination-reinitiation (stop-start) mechanism, during which the polymerase remains bound to the template. Transcription yields a series of sub-genomic monocistronic mRNAs. The stop-start mechanism is thought to be directed by short conserved sequence elements found at gene boundaries. For VSV and SeV, these include i) the gene-start (GS) motif at the upstream border of each gene, which encodes the precise 5' mRNA end and is assumed to be involved in mRNA initiation ii) the gene-end (GE) motif at the downstream border of each gene, which encodes the precise 3' end of mRNA, is assumed to direct transcriptional termination and is thought to direct polyadenylation by reiterative copying of the short tracts of U residues at the downstream end of the signal.

Similarly for RSV, consensus GS and GE motifs were identified at the begining and end of each gene which were shown to encode the precise 5' and 3' ends of mRNA with the latter being followed by a polyA tail (Collins *et al*, 1996b: Collins, 1991b). The RSV GS and GE motifs were identified as signals which direct transcriptional initiation and termination / polyadenylation respectively. They appear to operate independently to switch the polymerase into 'on' and 'off' modes respectively, and in which the polymerase moves along the genome with and without RNA synthesis respectively. In the 'on' mode, triggered by the GS motif, the polymerase transcribes until it encounters a GE signal. In the 'off' mode, triggered by the GE signal, the polymerase moves along the genome until it encounters the next GS signal and synthesis restarts.

However, during genome replication, the polymerase somehow switches to a read-through mode in which these GS and GE signals are ignored and a complete positive sense replicative intermediate, the anti-genome, is generated (Kuo *et al*, 1996).

Other *cis*-acting elements which might be involved in transcription and / or replication have been proposed. Studies on the 3' end of human parainfluenza virus type 3 (hPIV3) indicated that the promoter-proximal gene contained an additional *cis*-acting element located in the non-translated region following the GS motif (Collins *et al*, 1993, Dimock *et al*, 1994). Whereas in measles virus, terminal complementarity of the genome suggested the presence of an additional conserved element located at 75-90 nucleotides from the 3' ends of the genome and antigenome (Crowley *et al*, 1988).

Mutational analysis of the VSV genome also suggested that the amount of terminal complementarity can influence transcription and RNA replication (Wertz *et al*, 1994). However, for a SeV DI, replication efficiency was affected by the promoter end primary sequence, but this was found to be independent of terminal complementarity (Tapparel and Roux, 1996). This finding was recently supported by a rival VSV study which concluded that the presence of specific sequences, rather than the extent of terminal complementarity, was a major determinant of replication efficiency (Li and Pattnaik, 1997).

There are also sequences found after one GE and before the next GS, known as intergenic regions which are variable in length for the *Rubulaviruses* and *Pneumoviruses* , but are exactly 3 nucleotides long in both the *Parainfluenzaviruses* and the *Morbilliviruses*. The influence of these intergenic regions on the polymerase complex varies from virus to virus due to the diversity of the intergenic sequences. It seems likely that the consensus-type intergenic regions of the *Parainfluenzaviruses* and the *Morbilliviruses* are part of the *cis*-acting sequence elements that guide transcription, as suggested by their conserved nature and close proximity to the GS and GE signals. Moreover, a higher level of readthrough mRNA is associated with naturally occurring nucleotide differences in VSV and SeV (Masters and Samuels, 1984; Gupta and Kinsbury, 1985), suggesting an effect on the activity of transcription signals. The nonconsensus intergenic regions of the *Rubulavirus* and *Pneumovirus* genera may play a variety of roles in gene regulation. For example, they may contain *cis*-acting sequences thus far unidentified or intergenic length may control transcriptional attenuation. However, studies on the intergenic regions of RSV showed that they had no effect on replication or transcription of a dicistronic RSV minigenome (Collins *et al*, 1996a).

1.4.3 Genome Replication

As in viral transcription, genome replication is initiated by the viral polymerase complex binding to the 3' extracistronic region. However, during replication, the

polymerase ignores all the GS and GE signals which give rise to the specific mRNAs during transcription, and instead, generates a complete positive strand, replicative intermediate, the antigenome. Like the genome, the antigenome is encapsidated but cannot itself act as a template for mRNA synthesis (Portner, 1982; Carlsen *et al*, 1985). Instead, it acts as a template for the generation of further genomic sense RNA (Reviewed in Galinski and Wechsler, 1991). Genome synthesis from antigenome templates is thought to take place in a similar fashion to that for antigenome synthesis. Since both genomic and antigenomic RNAs are encapsidated in a ribonucleocapsid complex (RNP), and constant protein synthesis is a prerequisite for replication of all negative-strand RNA viruses, it is assumed that RNA polymerisation and encapsidation with nucleocapsid protein are mechanistically linked. The genomic 3' region simultaneously represents the promoter and the 'encapsidation' signal, and therefore a mechanism must exist which prevents the encapsidation of mRNAs. In non-segmented viruses, this may be achieved through the release of the leader RNA carrying the encapsidation signal. Reinitiation at the junction between the leader and the first protein-coding gene appears to be necessary to convert the polymerase to transcription mode in which elongation is independent of assembly with NP protein (Vidal and Kolakofsky, 1989). For VSV, the site for initiation of antigenome encapsidation has been mapped to the first 14 nucleotides of the leader sequence (Blumberg *et al*, 1983). When proposing a replication model, one has to take into consideration that replication and encapsidation are concurrent, and involve the participation of preformed NP-P and P-L complexes (Horikami *et al*, 1992). A possible replication model is shown in Fig. 10 where the moveable polymerase complex (P-L) is associated with the RNP template via an interaction of P with assembled NP. Soluble NP protein is prevented from self-aggregation by forming an NP-P complex. During replication, P delivers NP to the site of RNA synthesis, possibly by interaction with a second P binding site on L, facilitating concominant RNA encapsidation. This step would rely on release of the P molecule after NP delivery. Both V and W proteins, also products from the P gene (see 1.3.2.3 for details), can bind soluble NP (Randall and Bermingham, 1996; Horikami *et al*, 1996)

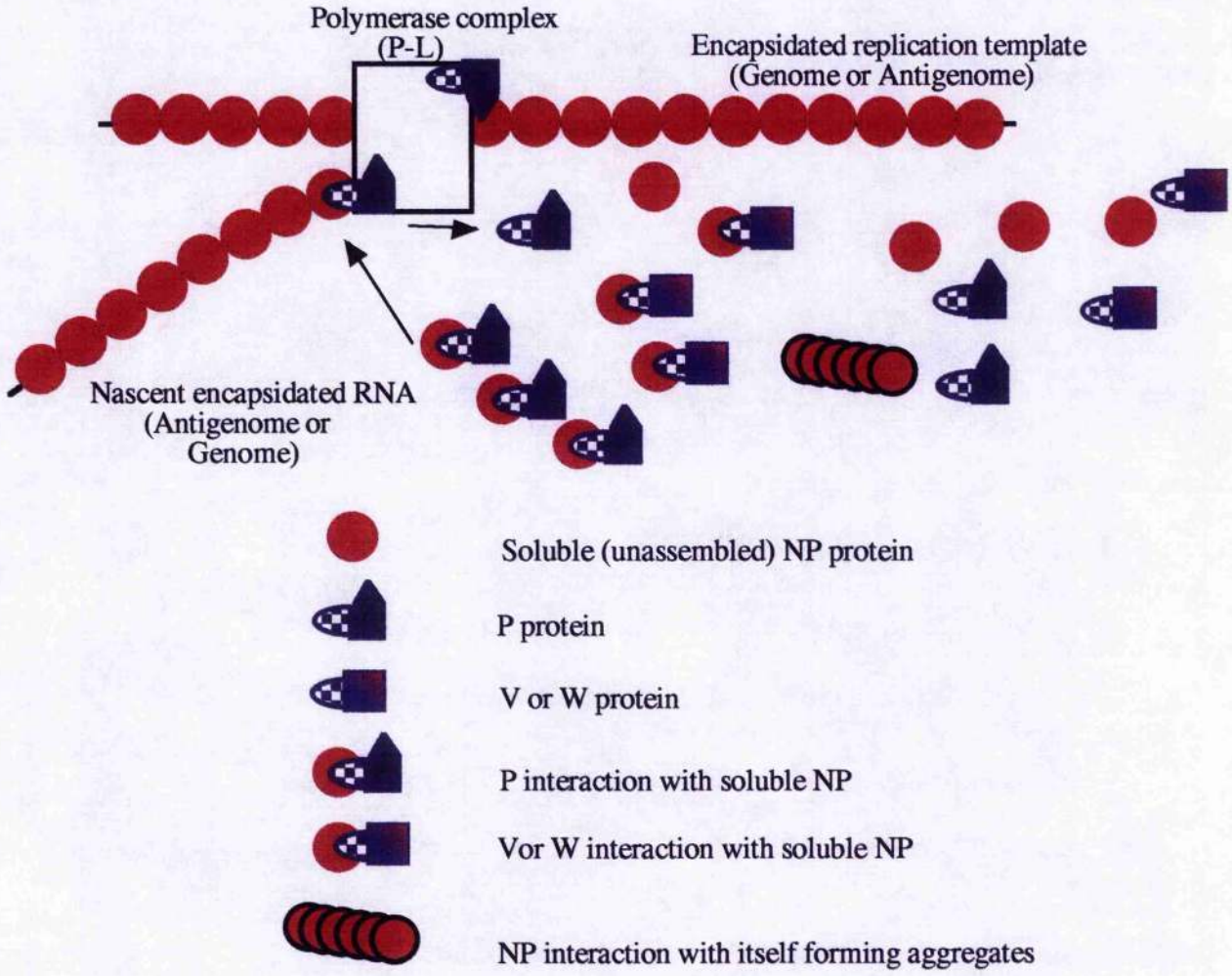


Fig. 10 Model for paramyxovirus replication

P-L polymerase complex is anchored to the RNP via P-RNP complexes. P binds to soluble NP protein preventing NP aggregation and subsequently chaperones NP to the nascent RNA for assembly. Both V and W proteins can bind soluble NP and can therefore inhibit replication by either preventing formation of, or binding by the NP-P encapsidation substrate.

and presumably inhibit replication by interfering with the formation of the NP-P encapsidation substrate (Horikami *et al.* 1996).

1.4.4 Virus Maturation and Release

Once synthesised, the nucleocapsid is transported to the plasma membrane for assembly into a virus particle. The conventional model for virus assembly is that M protein interacts with the viral glycoproteins via their cytoplasmic or transmembrane domains. An M protein with a glycoprotein spike attached, may diffuse in the plane of the plasma membrane until it contacts another M protein, and so on. A patch of M proteins forms on the cytoplasmic face of the membrane with its associated glycoprotein spikes on the external plasma membrane. Shortly thereafter, free nucleocapsids associate with the plasma membrane-bound M protein whereupon the budding process begins (Peeples, 1991).

Virions form by budding or outfolding through areas of cell membrane containing glycoprotein spikes. The virions acquire a lipid bilayer from the host cell in this highly specific process of virus assembly, during which host cell proteins are effectively excluded from the virus. Virions are also devoid of neuraminic acid residues due to the presence of neuraminidase activity in the virus which aids release (Reviewed by Ray *et al*, 1991).

2 Genetic Manipulation of Non-Segmented Negative-Strand RNA viruses

Recombinant DNA techniques have been used extensively to genetically manipulate DNA viruses and many RNA viruses. These studies have contributed to a better understanding of the molecular biology of virus infection, replication and pathogenicity, and have enabled the engineering of viral vectors suitable for the expression of foreign proteins in host cells. Several DNA viruses, including vaccinia viruses, herpes simplex viruses and adenoviruses, have been used to express foreign

proteins in cell culture, while many have potential as vaccine vectors or for the transfer of novel genes to target cells.

Recombinant DNA technology has also led to the construction of infectious full length cDNA clones of positive-strand RNA viruses such as picornaviruses (Racaniello and Baltimore, 1981) and bacteriophages (Taniguchi *et al*, 1978). Specific mutagenesis of these cDNA clones has provided important insights into the molecular biology of these viruses. In addition, many of these viruses, such as poliovirus (Percy *et al*, 1992) and Semliki Forest virus (Liljestrom and Garoff, 1991), can be genetically manipulated to express foreign genes and are therefore being developed as virus vectors. The RNA of positive-strand viruses can be used directly as mRNA and so RNA transcription *in vitro* of a linearized full length cDNA copy of the genome, results in infectious RNA molecules. These genomic RNA molecules can be transfected directly into the appropriate host cell to initiate productive virus infections via translation of the input RNA, thus generating the proteins responsible for both amplification and packaging of the progeny virions.

However, genetic manipulation and analysis of negative-strand RNA virus biology has lagged far behind DNA viruses and indeed that of other RNA viruses. In contrast to the positive-strand RNA viruses, the genomes of the negative-strand viruses are not infectious. The initiation of the infectious cycle requires the presence of a complete nucleocapsid structure. Only in this form can the RNA function as a template for the viral polymerase. Techniques to introduce recombinant RNA into the genome of a negative-strand RNA virus were first described for the segmented influenza virus (reviewed in Garcia-Sastre and Palese, 1993), and these techniques have been adapted to study *cis*- and *trans*-acting elements involved in paramyxovirus transcription and replication.

2.1 Reverse Genetics Approaches

Luytjes *et al* (1989) described a system that allowed the successful generation of biologically active influenza virus RNPs which contained artificial RNA. Genomic

sense transcripts were generated which contained authentic terminal sequences from an influenza virus segment, flanking the chloramphenicol acetyltransferase (CAT) reporter gene. These transcripts were encapsidated *in vitro* by purified virus necleocapsid protein (NP) and polymerase proteins (PA, PB1 and PB2). Once encapsidated, the synthetic nucleocapsid was transfected into influenza virus infected cells and was replicated, transcribed and translated by the helper virus. This breakthrough heralded influenza virus accessible to experimental investigations addressing the role of *cis*-acting sequences as well as the functions of several proteins in virus-host interactions. This work aided similar attempts in non-segmented RNA virus systems. In contrast to influenza virus, *in vitro* encapsidation of the non-segmented RNA genome is ineffective, probably because of the tighter RNP structure (Baudin *et al*, 1994). Only a few nucleotides corresponding to the genome ends of VSV could be associated *in vitro* with RNP proteins (Moyer *et al*, 1991; Smallwood and Moyer, 1993). However, these studies showed that functional encapsidation of preformed RNAs from non-segmented viruses was possible in principle, and confirmed the location of the RNA encapsidation signal close to the 3' end of the genome.

Further work was then done to encapsidate a synthetic RNA into a biologically active nucleocapsid and success was first reported for SeV by Park *et al* (1991). The synthetic RNA was generated *in vitro* by T7 RNA polymerase from a linearised plasmid, in order to create a precise 3' end for encapsidation. This genome analogue possessed authentic SeV 3' terminal sequences (which included the putative polymerase promoter and signal(s) directing leader RNA transcription / release and mRNA transcription initiation) and 5' trailer region (which included the transcriptional stop / polyadenylation signal drived from the 5' terminal L gene, and antigenomic replication promoter) as shown in Fig 11. The RNA transcript was transfected into SeV infected cells for encapsidation, and the resultant synthetic RNP had the potential to act both as a template for mRNA synthesis and as a template for replication. This study demonstrated that transfection of the *in vitro* transcribed RNA into SeV infected cells, led to the detection of CAT activity which was shown to by SeV specific. Furthermore, CAT activity was sucessfully passaged by transfer of cell-free supernatants,

demonstrating that the synthetic RNPs had been packaged into infectious virus particles. This confirmed that all *cis*-acting sequences required for encapsidation and initiation of both replication and transcription of SeV, resided in the terminal sequences of the genome.

Other paramyxovirus systems have also been successful in rescuing transfected RNAs with infectious helper virus. These included RSV (Collins *et al*, 1991b), PIV3 (De and Banerjee, 1993; Dimock and Collins, 1993) and MeV (Sidhu *et al*, 1995), where assays of reporter gene activity expressed from a negative-sense minigenome, again indicated that they were transcribed, replicated and incorporated into infectious particles. The roles of *cis*-acting sequences in paramyxovirus transcription and replication had therefore been rendered accessible to experimental investigation. Subsequent experiments revealed that the sequence corresponding to the virus 3' terminus, had to be at the end of the synthetic transcript, as replication cannot proceed from an internal site (Collins *et al*, 1991b; De and Banerjee, 1993). In contrast, the 5' end appeared to tolerate additional nucleotides (De and Banerjee, 1993). Furthermore, for RSV it was shown that sequences of the 3' promoter could be replaced by sequences complementary to the 5' end (ie the antigenome promoter; Collins *et al*, 1991b). Similar reporter gene constructs have been used to study the roles of intergenic regions as internal transcription signals, where two different reporter genes, flanked by the 3' and 5' viral sequences described above, were seperated by the intergenic region of interest (Kuo *et al*, 1996).

2.2 RNPs derived from cDNA components

Defective interfering (DI) particles have been extensively used to investigate transcription mechanisms of the negative-strand RNA viruses. Most are naturally occurring and can be catagorised as non-transcribing copyback DIs, since they contain the parental 5' terminus and complementary 3' end (Fig. 11). Therefore, these DIs are replication competent but cannot initiate transcription and have been used to elucidate *trans*-acting factors necessary for their propagation.

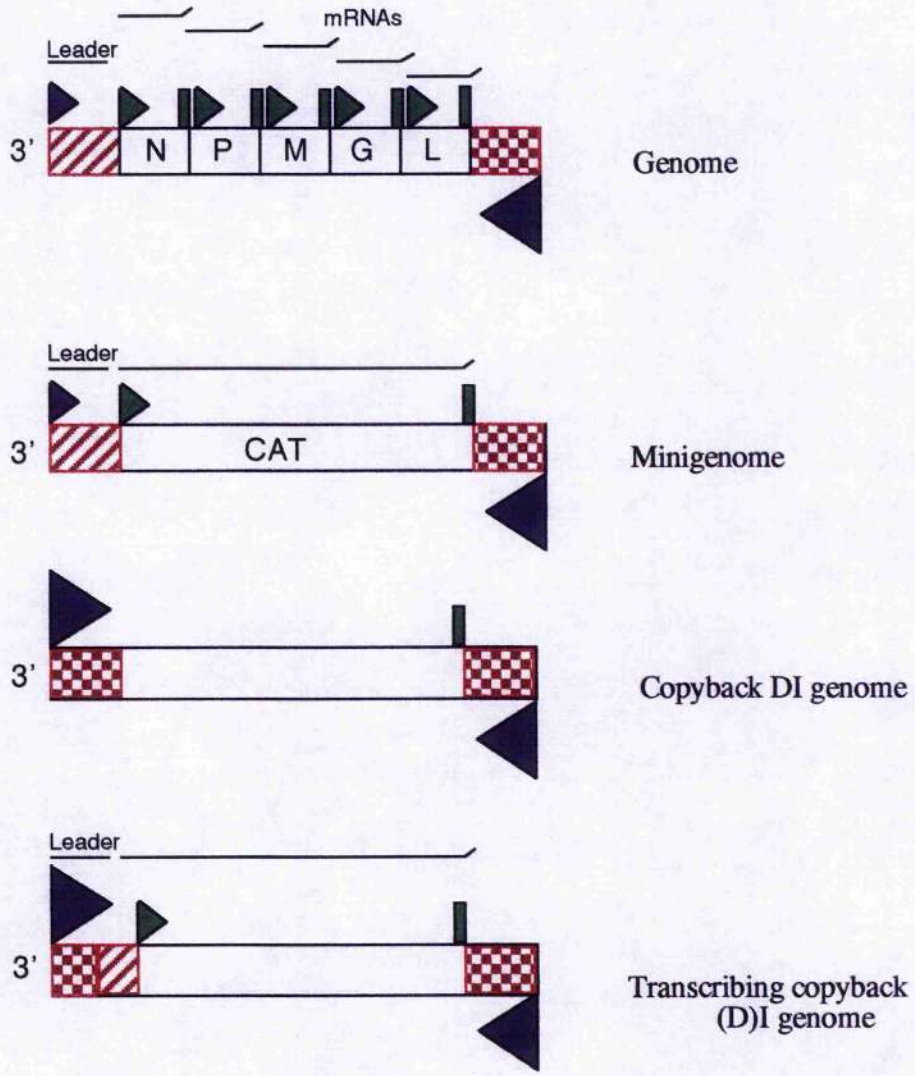


Fig.11 Schematic diagram of genome analogues

Schematic representation of the organisation of genomes and genome analogues from non-segmented negative-strand RNA viruses. Terminal non-coding regions of the standard genome are hatched (3') or checked (5'), polymerase promoters are represented by blue arrowheads (genome promoter above, and antigenome promoter below the genome). Transcriptional restart signals are represented by green arrowheads, and transcription stop/polyadenylation signals by green bars above the genomes. The resultant transcrition products are indicated. In copyback genomes, the 3' terminal sequences are replaced by the complement of 5'-terminal sequences. Both copyback and transcribing copyback genomes (either defective or non-defective) interfere with the replication of standard genomes.
Adapted from Conzelmann (1996).

NP, P and L proteins were demonstrated to be necessary and sufficient to replicate a VSV DI (Pattniak and Wertz, 1990) and a SeV DI (Curran *et al*, 1991). These proteins were supplied *in trans* by transfecting cDNA clones of NP, P and L under the control of a T7 promoter, which were driven by a recombinant vaccinia virus expressing T7- RNA polymerase (vTF7-3; Fuerst *et al*, 1986). Furthermore for VSV, if the cells co-infected with vTF7-3 and VSV DI, also expressed the envelope proteins (in addition to NP, P and L) from plasmids, then assembly and budding of an infectious virus was obtained (Pattnaik and Wertz, 1991). This allowed helper virus to be replaced by cDNA clones in rescue experiments.

2.2.1 The Use of vTF7-3 for the Generation of Genome Analogues

The use of vTF7-3 (VacT7) allowed the expression of a number of viral proteins from different co-transfected plasmids. It also allowed the generation of intracellular genome analogues as a replacement for the *in vitro* transcribed templates described in section 2.1. cDNA clones encoding the genome analogues under T7 promoter control, could be co-transfected with those encoding the viral proteins into VacT7 infected cells. Thus, simultaneous generation of intracellular genome analogues and viral proteins could be achieved. The termini of the genome analogues were paramount. The 5' end was determined by the positioning of the T7 promoter, while the autolytic activity of ribozyme sequences were utilised to generate a discrete 3' terminus. The ribozyme sequences were usually derived from the antigenomic strand of the hepatitis delta virus (HDV; Perrotta and Been, 1990, 1991) and were followed by a T7 transcriptional termination sequence. Only sequences downstream of the cleavage site seem to be required for auto-catalytic activity, as the ribozyme is aparently indiscriminate with regard to the upstream sequences.

A VSV DI sequence was cloned between the T7 promoter and ribozyme sequences for the generation of a synthetic DI. This plasmid was co-transfected with those of NP, P and L into VacT7 infected cells and resulted in the efficient encapsidation and replication of the synthetic DI RNA (Pattnaik *et al*, 1992).

2.2.2 Transcriptionally Active Genome Analogues

An RNA construct corresponding to a transcriptionally active internal deletion minigenome of the *Rhabdovirus* rabies virus, was the first to be rescued by plasmid-encoded (NP, P and L) proteins alone (Conzelmann and Schnell, 1994). As seen with VSV, infectious DI virions of rabies virus were assembled after addition of the envelope proteins, thus opening all aspects of the life-cycle to experimental analysis. This breakthrough led to similar constructs for several non-segmented viruses (VSV, SeV, MeV, RSV), corresponding to transcribing or non-transcribing model genomes, expressed in the VacT7 / HDV expression system (Reviewed in Conzelmann, 1996). These studies revealed virus- and genus-specific peculiarities which have to be taken into account when designing reverse genetics experiments. For example, SeV virion assembly is inhibited by vaccinia virus (Calain and Roux, 1993) whereas the entire life-cycle of rabies virus is unaffected by the presence of vaccinia virus. These authors have also reported that model genomes of SeV which consist of a multiple of six nucleotides, replicate more efficiently than those that do not (Calain and Roux, 1993, Pellet *et al*, 1996). This has been explained by the NP protein having been predicted to cover a stretch of 6 nucleotides in the RNP and is known as ' The Rule of Six '. It seems to apply to the *Morbillivirus* MeV (Kalin *et al*, 1994), and the *Rubulavirus* SV5 (Murphy and Parks, 1997) but not the *Pneumovirus* RSV (Samal and Collins, 1996) or rhabdoviruses. However, NP, P and L proteins seem to be necessary and sufficient for both transcription and replication of all of these minigenomes (e.g. Yu *et al*, 1995; Grosfeld *et al*, 1995). In addition to these proteins, some paramyxoviruses may express auxilliary proteins which regulate RNA synthesis. For SeV, V and W proteins have been shown to inhibit genome replication (Curran *et al*, 1991, Horikami *et al*, 1996) whereas the RSV M2 protein seems to have a positive effect on mRNA transcription (Grosfeld *et al*, 1995). Reverse genetics approaches using model genomes will provide a positive tool for the investigation of mechanisms involved in paramyxovirus biology.

2.3 Recovery of Infectious Virus from cDNA clones

Using the techniques described above, the generation of a full-length infectious clone entirely from cDNA was theoretically possible. One main stumbling block remained. The encapsidation efficiency of long RNA molecules was very low and in the rabies virus system, each additional kilobase of RNA in the model genome, resulted in a 10-fold drop in recovery rate (Conzelmann and Schnell, 1994). However, a 50% full-length genome analogue of RSV was described, which contained the entire RSV L gene and the CAT reporter gene, and was rescued by transfection of the synthetic transcript into RSV infected cells.

The generation of a full length infectious clone from the VacT7 system described above had an additional problem. A synthetic full-length genome-sense clone would contain the NP, P and L coding sequences. Large amounts of the mRNAs encoding the NP, P and L proteins which are required for encapsidation, replication and transcription of the synthetic transcript would be expected to hybridize to the negative sense RNA template thūs blocking proper encapsidation and / or synthesis of the necessary proteins.

To overcome these problems, a cDNA clone for rabies virus was constructed which gave rise to an antigenomic (positive) sense RNA transcript. This transcript was encapsidated and replicated by plasmid-encoded proteins. Transcription was achieved from the resultant genomic RNA template, initially also by the plasmid-encoded proteins, and then by autonomous propagation of recombinant virus (Schnell *et al*, 1994). Although rescue efficiency was low (occurring in only one per 10^7 cells), this did not constitute a difficulty as the system worked in the absence of helper virus, allowing the isolation of variants with titres much lower than the parental strain. Vaccinia virus was easily removed by filtration or by passage in non-permissive cells, allowing the isolation of pure clonal virus stocks.

Recent successful recovery of other recombinant viruses confirmed that the use of antigenomic rather than genomic transcripts may generally tip the balance towards success. Two groups working with VSV found recovery was not possible when starting with plasmids yielding genome sense transcripts but recovery of full-length antigenome RNA was reported (Lawson *et al*, 1995; Whelan *et al*, 1995). One reason for their

failure was suggested by Whelan *et al* (1995) as they found premature termination of transcription by T7 RNA polymerase in *in vitro* transcription experiments.

The first recovery of a recombinant paramyxovirus was achieved for MeV in a similar fashion to rabies virus (Radecke *et al*, 1995). Here, cell lines constitutively expressing T7 RNA polymerase and the virus NP and P proteins, were transfected with additional plasmids driven by the T7 promoter. These plasmids encoded MeV L protein and a full-length MeV antigenome RNA. The appearance of syncytia demonstrated rescue of infectious virus.

Recombinant SeV was also recovered from a full-length antigenomic transcript using the VacT7 system, despite vaccinia virus inhibition of SeV virion assembly. This inhibition was overcome by treating the transfected cells with an inhibitor of DNA synthesis, injection of the transfected cells into the allantoic cavity of embryonated chicken eggs and subsequent passage in eggs, resulting in the loss of VacT7 and recovery of infectious SeV (Garcin *et al*, 1996).

Genetic manipulation of the negative strand viruses has now become a reality for both the segmented viruses, such as influenza virus, and for the non-segmented viruses within the rhabdo- and paramyxovirus families. Recombinant viruses can now be used to study the *cis*-acting sequences controlling viral RNA synthesis as well as the function of each virus protein in replication, assembly and interaction with the host, thus, fundamentally changing this field of virology.

3 Aims of this project

3.1 Development of a Reverse Genetics System for SV5

The initial aim of the project, which was begun in 1992, before reverse genetics was well established for non-segmented, negative-strand RNA viruses, was to elucidate the mechanisms of transcription and replication of the *Rubulavirus* SV5 by utilising some of the reverse genetics techniques outlined above. A synthetic genome sense RNA transcript, containing the CAT reporter gene, was used as the template for rescue

experiments. Three approaches were employed which aimed to encapsidate the synthetic transcript into an RNP recognisable to the viral polymerase as a transcription template. The first involved intracellular transcription of the synthetic RNA by VacT7 in SV5 infected cells. It was hoped that SV5 would supply the viral proteins necessary for encapsidation and transcription of the synthetic RNA. The second approach involved transcfection of the synthetic transcript into cells expressing plasmid-encoded viral proteins. Here, it was hoped that the viral proteins supplied *in trans* would encapsidate and transcribe the synthetic transcript. The third approach involved transfecting the synthetic RNA into a cell line co-expressing NP, P and V proteins for encapsidation, with the viral polymerase being supplied by a subsequent SV5 helper virus infection. It was hoped that once the transcript was encapsidated, the viral polymerase would recognise the synthetic RNP as a template for transcription and possibly replication.

3.2 Development of Inducible Cell Lines Expressing Viral Proteins

Mammalian cell lines expressing NP, P and V proteins of SV5, either singly or in combination, were isolated with the aim of supplying the viral proteins *in trans* for CAT rescue experiments outlined above. The cell lines subsequently proved extermely useful for examining viral protein:protein interactions. The change in intracellular distribition of the viral proteins was monitored by comparing the immunofluorescence patterns of the proteins expressed alone and in combination, indicating viral protein:protein interactions were taking place.

3.3 Viral Protein : Protein Interactions

A novel protein : protein capture assay was developed to examine more closely the viral protein interactions and perhaps understand more clearly the roles these interactions play in viral transcription and replication. Results from both the immunofluorescence

and the capture assay studies gave some insight into, and possible explanation for, the lack of success of the SV5 reverse genetics / infectious clone project to date.

Chapter 2 : Materials and Methods

1 Cells and viruses

1.1 Maintenance of mammalian cell lines

The following cell lines were used during the course of this study.

Vero cells - a continuous line of African Green Monkey kidney cells (Flow Labs)

BHK-21 Cl.13 - (referred to as BHK) are a continuous line of baby hamster kidney cells (Flow Labs)

HeLa cells - a continuous cell line of human cervical carcinoma cells (Flow Labs)

Balb/c mouse fibroblasts - (referred to as BalbC) are a continuous line of fibroblasts cloned from a primary culture of a Balb/c mouse embryo.

293 cells - human cell line transformed by human adenovirus type 5 and generously provided by Frank Graham, McMaster University, Hamilton, Ontario, Canada (Graham *et al,* 1977).

Cell lines were routinely grown in Glasgow Modified Eagle's Medium (GMEM), (Gibco-BRL), supplemented with 10% (v/v) newborn calf serum (NBCS) or foetal calf serum (FCS), 50 units /ml of penicillin and 50 µg/ml streptomycin at 37 °C under 5% CO_2. Cells were passaged every 4 - 5 days using trypsin (BHK, HeLa, BalbC, Vero) or versene (293).

1.2 Preparation of SV5

1.2.1 Preparation of SV5 W3 working stock

The strain of SV5 used throughout this work was W3 (Choppin, 1964). A working stock was made by first plating $2x10^4$ cells per well of a 96 well dish. These cells were

infected with 50 µl of virus in a series of 5-fold dilutions starting from a one in ten dilution of a master virus stock prepared by Dan Young in this lab. The virus was diluted in GMEM+1% NBCS and 8 wells were infected at each dilution. The cells were incubated at 37 °C until the cytopathic effect (CPE) caused by the virus was obvious. The medium from the well with most complete cell lysis was transferred onto a confluent 25 cm ^{2} tissue culture flask of Vero cells. The cells were incubated again until CPE was obvious, whereupon the medium was used to infect 4 confluent roller bottles of Vero cells. Here, the virus was adsorbed for 2 hours at 37 °C and then the inoculum was replaced with 20 mls GMEM+1% NBCS. The roller bottles were rotated at 37 °C for 36-40 hours or until the CPE was again obvious. The supernatant was collected and the cell debris was pelleted by centrifugation at 5,000 x *g* for 5 mins. The supernatant containing the virus was aliquotted in 5ml and 1 ml amounts and stored at -70 °C. Titration of the new virus stock was done by plaque assay (1.2.2). Both cells and virus were examined for mycoplasma contamination by DAPI staining (Russell *et al,* 1975), (1.4).

1.2.2 Plaque assay titration of SV5 stock

Vero cells were seeded in 6 well dishes at $5x10^5$ cells per well and incubated overnight (O/N). A series of 10-fold dilutions were then made of the new virus stock in GMEM+10% NBCS. 1 ml of virus from 10^{-4}, 10^{-5} and 10^{-6} dilutions were used to infect the plate in duplicate. Virus was adsorbed to the cells for 2-3 hours at 37 °C in a gassed box set on a rocking platform. The inoculum was removed and replaced with 5 mls of GMEM containing 2% (v/v) NBCS and 0.5% (w/v) carboxymethyl cellulose whereupon the cells were incubated for 4-5 days in a 37 °C incubator under 5% CO_2. The cells were then fixed by the addition of 2.5 mls of a PBS solution containing 5% (v/v) formaldehyde and 2% (w/v) sucrose for 10 mins. The fixing solution was removed by aspiration and the cells were washed in PBS then permeabilized for 5 mins in a PBS solution containing 0.5% (v/v) NP40 and 10% (w/v) sucrose. The cells were then washed three times in PBS containing 1% (v/v) NBCS and 0.1% (v/v) sodium azide (NaAz). Areas of cell lysis and therefore sites of virus infection or plaques were

detected by incubating the monolayers with a panel of monoclonal antibodies (mAbs) in 0.5 mls of PBS+1% NBCS (1/500 dilution of each mAb) per well for 1 hour on a shaker at room temperature (RT). The cells were then washed three times in PBS+1% NBCS, twice in PBS and once in dH_2O whereupon an ELISA reaction was carried out using o-dianisidine. Approximately 0.3g of o-dianisidine was dissolved in 0.5 mls of 100% ethanol, added to 50 mls of dH_2O and filtered through Whatman No.1 filter paper to remove any undissolved o-dianisidine. 0.6% (v/v) hydrogen peroxide was added to the filtrate and mixed well. 2mls per well was added to the cells which were incubated in the dark on a shaker until brown plaques were obvious. The reaction was stopped by washing in tap water and plaques were counted to give a virus titre of plaque forming units (pfu) per ml of stock. This was typically found to be $1x10^7$ to $1x10^8$ pfu / ml.

1.2.3 Infection of mammalian cells with SV5 W3

Typically BHK, BalbC or 293 cells were plated at $1x10^6$ cells per well in 6 well dishes and incubated O/N or until the monolayer was 70-80% confluent. Cells were infected at a multiplicity of infection (m.o.i.) of 1, (i.e. 1 pfu/cell), in GMEM+1% NBCS. The virus was adsorbed to the cells by incubation at 37 °C in a gassed box on a rocker for 2 hours. The inoculum was removed and replaced by GMEM+1% NBCS and harvested 36-48 hours post-infection.

1.3 Preparation of vaccinia virus vTF7-3 (VacT7)

1.3.1 Small scale preparation of VacT7

A recombinant vaccinia virus expressing the bacteriophage T7 DNA-dependent RNA polymerase (vTF7-3) was constructed by Fuerst *et al* , (1986) and is usually referred to here as VacT7. Small scale virus preparations were made by infecting a confluent 25 cm^2 tissue culture flask of HeLa cells at a m.o.i. of 1 with the master virus stock (made by Bernie Precious in this lab) in GMEM+1% NBCS. The virus was adsorbed to the cells for 1-2 hours in a 37 °C incubator whereupon the inoculum was removed and 5

mls of fresh GMEM+1% NBCS was added. The cells were incubated at 37 °C for a further 48 hours and harvested by scraping into the medium. Cells were pelleted by centrifugation at 3,000 x *g* for 5 mins and the pellet was resuspended in 2 mls of 10 mM Tris-HCl pH 7.0, 1 mM EDTA and the virus was released by six freeze / thaw cycles at - 70 °C and 37 °C. This preparation was used as the inoculum for large scale virus preparation.

1.3.2 Large scale production of VacT7

Ten 75 cm^2 tissue culture flasks were seeded with HeLa cells and grown to 90% confluency for infection with VacT7. The 2 mls of virus from a small scale preparation of VacT7 (1.3.1) was added to 18 mls of GMEM+1% NBCS and 2 mls of the dilution was added to each confluent monolayer. The virus was adsorbed for 1-2 hours at 37 °C on a rocker whereupon the inolculum was removed and replaced with 20 mls of GMEM+1% NBCS and grown for 24-48 hours. Cells were again scraped into the medium and pelleted by centrifugation at 3,000 x *g* for 5 mins. Cell pellets were resuspended in a total volume of 2 mls of 10 mM Tris-HCl pH 7.0, 1 mM EDTA and pooled into one tube. Cells were subjected to 5 freeze / thaw cycles at -70 °C and 37 °C and were aliquotted in 10 µl and 100 µl amounts for storage at -70 °C. Titration of the VacT7 stock was done by plaque assay (1.3.3).

1.3.3 Plaque assay titration of VacT7 stock

Titration of new large scale preparations of VacT7 (1.3.2) was done by plaque assay. 293 cells were seeded at $1x10^6$ cells per well in duplicate 6 well dishes and grown O/N or until 90% confluent. The virus was diluted in a series of 10-fold dilutions in GMEM+1% NBCS and 1 ml of the dilutions from 10^{-4} to 10^{-9} was added to the duplicate plates and adsorbed for 1-2 hours at 37 °C. The inoculum was removed and replaced with 2.5 mls of GMEM+1% NBCS and the cells were incubated for a further 48 hours at 37 °C. The medium was removed and the monolayer was stained with a solution of 0.1% (w/v) Crystal Violet, 2% (v/v) ethanol in dH_2O for 2-3 mins at RT. Areas of cell lysis caused by virus infection (plaques) were visualised as unstained

holes in the monolayer and were counted to determine virus titre in pfu /ml of stock. This was typically found to be $1x10^8$ to $1x10^9$ pfu / ml.

1.3.4 Infection of mammalian cells with VacT7

Routinely mammalian cells were infected at a m.o.i. of 1 (i.e. 1 pfu / cell) in a 1 ml volume of GMEM+1% NBCS per well of a 6 well dish. The virus was adsorbed to the cells for 1 hour at 37 °C under 5% CO_2 whereupon the inoculum was removed and replaced with 2.5 mls of GMEM+1% NBCS until harvesting. Normally cells were transfected (2.3.2) at 1 hour post-infection and harvested at 18 hours post-infection. For 90 mm diameter tissue culture dishes, infection at m.o.i. of 1 was carried out in 2.5 mls of GMEM+1% NBCS and incubated with 5 mls of GMEM+1% NBCS until harvesting.

1.4 DAPI staining of cells and virus

Cells were seeded onto multispot slides (C.A. Hendley, (Essex) Ltd) at a density of $1x10^4$ cells per well and were incubated O/N or until slightly sub-confluent. The medium was removed and the slides were washed in 100% methanol. Each well was treated with 50 µl of a 1 mg/ml solution of 4, 6, diamidino-2-phenyl-indole (DAPI) in methanol for 15 mins at 37 °C. The cells were then washed again in methanol, dried and coverslips were mounted on the slides in Citifluor (Citifluor Ltd) for examination by immunofluorescence microscopy. Uncontaminated cells had a fluorescent nucleus but no visible cytoplamic fluorescence. Mycoplasma contaminated cells exhibited cytoplasmic and extracellular fluorescence. By infecting an uncontaminated cell line with a new virus stock and staining the infected cells as described above, mycoplasma contamination of the virus stock was monitored.

2. Transfection of mammalian cells

2.1 Calcium phosphate mediated transfection

2.1.1 Preparation of BBS for transfection

BES-buffered saline (BBS) was prepared as described by Chen and Okayama (1987) for use in calcium phosphate mediated transfection. BBS was prepared in 400 ml batches of a 2x stock solution consisting of 50 mM BES (2-[bis(2-hydroxyethyl)]-2-amino-ethanesulphonic acid; 2-[bis(2-hydroxyethyl)amino]-ethanesulphonic acid),(SIGMA), 280 mM NaCl, 1.5 mM Na_2HPO_4. The pH of the solution was adjusted to pH 6.95 before filtering through 0.45 µm disposable filter and aliquotting in 50 ml amounts for storage at -20 °C.

2.1.2 Preparation of HBS for transfection

HEPES-buffered saline was prepared as a 2x stock consisting of 1.0g of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid), (SIGMA), 0.2g Glucose, 1.6g NaCl, 0.074g KCl and 0.025g $Na_2HPO_4.2H_2O$ per 100 mls of d H_2O. The pH of the solution was adjusted to 7.05 whereupon it was then filter sterilized through 0.45 µm disposable filter and stored at -20 °C until needed.

2.1.3 Transfection of mammalian cells with BBS or HBS

A common way of introducing DNA into mammalian cells is by calcium phosphate treatment. Although the mechanism remains obscure, it it thought that DNA enters the cytoplasm of the cell by endocytosis of the calcium phosphate - DNA precipitates, whereupon the DNA travels to the nucleus for processing. To utilise this method, cells were seeded in 6 well dishes at $1x10^6$ cells per well in GMEM+10% NBCS and incubated at 37 °C O/N or until cells were 80% confluent. The medium was replaced with 2.5 mls of fresh GMEM+10% NBCS at least 2 hours prior to transfection. For each well a 250 µl transfection cocktail was prepared as follows :- 125 µl 2xBBS or 2xHBS, appropriate DNA to be transfected, 12.5 µl of $CaCl_2$ (2.5 M filter sterilized)

and dH_2O to make the volume up to 250 µl. Note that the $CaCl_2$ was added last and mixed thoroughly to ensure formation of DNA-calcium phosphate complexes. Cocktails were incubated at RT for 20 mins and added dropwise onto the medium of the appropriate wells of cells. The medium was changed 16 hours post-transfection and cells were incubated for a further 24 hours before harvesting.

2.2 DEAE-dextran mediated transfection

Diethylaminoethyl-dextran (DEAE-dextran) method of transfection described by Luytjes *et al* (1989) was used. Cells were seeded in 6 well dishes at $1x10^6$ cells per well and incubated O/N or until cells were 80% confluent. Each well was treated with 1 ml of DEAE-dextran solution (300 µg/ml DEAE-dextran (Sigma), 0.5% (v/v) DMSO, in a PBS-gelatin solution (0.1 mg/ml gelatin)) for 30 mins at RT. The solution was aspirated off and replaced with DNA in a 200 µl volume of PBS-gelatin (0.1 mg/ml) and incubated for 1 hour in a 37 °C incubator. The transfection mix was replaced with 2.5 mls of GMEM+10% NBCS and the medium was changed 16 hours post-transfection. Cells were harvested 24 hours later.

2.3 Liposome mediated transfection

2.3.1 Preparation of cationic liposomes

Cationic liposomes equivalent to TransfectACE (Gibco-BRL) were prepared according to the method of Rose *et al*, (1991) with a weight:weight ratio of 1:2.5 DDAB:DOPE. 1 ml of a 10 mg/ml solution of DOPE (dioleoyl-L-α-phosphatidyl ethanolamine), (Sigma) in chloroform, was pipetted into a glass universal whereupon 4 mg of DDAB (dimethyldioctadecyl ammonium bromide),(Sigma), was added and dissolved. The choloform was evaporated off under a stream of nitrogen gas and the lipids were resuspended in 10 mls of sterile dH_2O by placing in a sonicating water bath for 30 mins. Once suspended, the cloudy lipid solution was clarified by sonication using a fine soniprobe for 30 second intervals at maximum power while keeping the universal on ice. The lipids were aliquotted in 500 µl amounts in small glass screw-capped tubes and

stored at 4 °C until needed. Due to potential batch variation, the transfection efficiency of each batch was monitored by β-galactosidase assay on cells transfected with a known amount of the plasmid pPEβGal (5.9).

2.3.2 Liposome mediated transfection

Cells were seeded at $1x10^6$ cells per well in 6 well dishes and incubated O/N or until 80% confluent. Nucleic acid was diluted to 100 µl in OPTIMEM® Reduced Serum Medium (Gibco-BRL) and combined with separately diluted Lipofectin ™ (Gibco-BRL) or liposomes made as in 2.3.1. (10 µl of liposomes + 90 µl of OPTIMEM® per well made in bulk so each well received 100 µl of the same liposome mix). The nucleic acid and lipid were incubated together at RT for 15 mins. During this incubation, cell monolayers were washed with 2 mls of OPTIMEM®. After the 15 min incubation, 800 µl of OPTIMEM® was added to each reaction. Medium was aspirated off the cell monolayers and replaced with 1 ml of lipid-nucleic acid mix and incubated O/N. The medium was changed at 16 hours post-transfection to 2.5 mls of GMEM+10% NBCS and incubated for a further 24 hours before harvesting.

3 Recombinant DNA Technology

3.1 Large scale production and purification of plasmid DNA

This method was used to prepare DNA for transfection, dideoxynucleotide sequencing, *in vitro* transcription, PCR, coupled *in vitro* transcription/translation and cloning. If the plasmid yield was poor, then the culture volume was increased to 500 mls and the appropriate volume adjustments were made.

A single bacterial colony was used to inoculate 5 mls of Luria Broth (LB) containing the appropriate antibiotic for selection (e.g. 100 µg/ml Ampicillin) and incubated at 37 °C overnight, (O/N), with shaking. 1ml of the O/N culture was then used to seed 50mls of LB + antibiotic, and grown for at least a further 12 hours at 37 °C with shaking. The culture was then centrifuged at 4,000 x *g* in a 50 ml screw-capped disposable (e.g.

Falcon) tube for 10 mins at 4 °C. The pelleted cells were resuspended in 4 mls of ice-cold TGE (25 mM Tris-HCl, pH 8.0, 50 mM Glucose, 10 mM EDTA). The bacterial cells were then lysed by addition of 8 mls of a 0.2 M NaOH, 1% SDS solution, shaken well to mix and incubated on ice for 5 mins. 6 mls of a 5 M potassium acetate solution (3 M with respect to potassium and 5 M with respect to acetate) was added to the lysate, shaken to mix and incubated on ice for 10 mins. Cellular debris was pelleted by centrifugation at 4,000 x *g* for 15 mins at 4 °C and the supernatant was filtered through a piece of muslin into a fresh tube. 17 mls of isopropanol was added to the filtrate and then chilled at - 70 °C for at least 1 hour. The tube was then centrifuged at 4, 000 x *g* for 15 mins at 4 °C whereupon the supernatant was discarded and the pellet was resuspended in 2 mls of TE (10 mM tris-HCl, pH 7.5, 1 mM EDTA). 2.5 mls of 4.4 M LiCl was added and then incubated on ice for at least 10 mins. The tube was centrifuged at 4,000 x *g* for 15 mins at 4 °C, the supernatant decanted into a fresh tube to which 10 mls of 100% ethanol was added and incubated for 15 mins at RT. After spinning again at 4,000 x *g* for 15 mins at 4 °C, the supernatant was discarded and the pellet was washed in 70 % (v/v in water) ethanol, resuspended in 400 µl of TE and transferred to a 1.5 ml eppendorf tube. The solution was treated with 4 units (2 µl) of DNase-free RNase (Boehringer-Mannheim) and incubated at 37 °C for 15 mins. The reaction was terminated by the addition of 20 µl of 10 % sodium dodecylsulphate (SDS) and heated to 70 °C for 10 mins, followed by two phenol extractions, two phenol/chloroform extractions (1:1 ratio v/v) and two chloroform extractions. The DNA was precipitated by the addition of one tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100 % ethanol, and incubated at RT for at least 15 mins. The DNA was pelleted by centrifugation in a benchtop centrifuge at 14,000 x *g* for 15 mins, washed in 70 % ethanol (v/v in water), dried and resuspended in 100 µl of TE. The concentration and purity of the DNA was confirmed by both optical density analysis at 260 nm and 280 nm and by restriction digests followed by electrophoresis through a 0.8 % (w/v) agarose gel (3.4). 1 absorbance unit at 260 nm is equal to 50 mg/ml of double stranded DNA and a comparison of the ratio of absorbance at 260 nm to 280 nm, gives an indication of protein contamination at 280 nm absorbance, and therefore the purity of the sample.

an indication of protein contamination at 280 nm absorbance, and therefore the purity of the sample.

3.2 Small scale production of plasmid DNA

Small scale plasmid production was usually carried out for the screening of recombinant clones after DNA cloning procedures. The clones were screened by restriction digest to verify that fragments of the expected molecular weight were released. Once a positive clone was identified, a large scale preparation was made. The alkali-lysis method used was based on that of Birnboim and Doly (1979). 5 mls of LB including the appropriate antibiotic was inoculated with a single bacterial colony and incubated overnight (O/N) at 37 °C with shaking. 1.5 mls of the O/N culture was transferred into a 1.5 ml eppendorf tube and centrifuged for 1 min at high speed in a benchtop microfuge. The supernatant was then aspirated off and the bacterial pellet was resuspended in 100 µl of ice cold TGE and then lysed with 200 µl of a freshly made 0.2 M NaOH, 1 % SDS solution, mixed by inverting 3 times and incubated on ice for 5 mins. 150 µl of potassium acetate solution (3 M with respect to potassium and 5 M with respect to acetate) was added and the tube was vortexed in an inverted position for 10 seconds. The tube was then incubated on ice for 5 mins and then centrifuged at high speed in a benchtop microfuge for 5 mins. The supernatant was transferred to a fresh eppendorf tube avoiding all the floating debris whereupon 2 volumes of 100 % ethanol were added and incubated at RT for at least 5 mins. DNA was precipitated by centrifugation at high speed in a benchtop microfuge for 10 mins, washed in 70 % ethanol (v/v in water), dried and resuspended in 50 µl of TE. 1 µl of miniprep DNA was used in a restriction digest reaction for analysis of DNA fragments by agarose gel electrophoresis.

3.3 Restriction digestion of plasmid DNA

Digestion of DNA by the appropriate restriction enzyme was carried out as part of a cloning procedure or screening procedure to verify DNA restricted as expected. For small scale plasmid preparations, typically 1-5 µl DNA was digested in a total reaction

volume of 20 µl which included buffers supplied by the maufacturer (Gibco-BRL or New England Biolabs) diluted to the appropriate concentration, 0.1 units of restriction enzyme and 0.2 units of DNase-free RNase (Boehringer Mannheim). Digestions were carried out from 1-4 hours at the appropriate temperature as stipulated by the manufacturer. Typically 1 µg of a large scale DNA preparation was digested in a 20 µl reaction volume containing the appropriate buffer supplied by the manufacturer, and 1 unit of restriction enzyme for at least 1 hour at the appropriate reaction temperature. If the DNA preparation was the vector backbone for use in a ligation reaction, its ends were dephosphorylated by the addition of 0.5 µl (12 units) of alkaline phosphatase (Boehringer Mannheim) for the last hour of the digest. The alkaline phosphatase was inactivated by the addition of EGTA to a final concentration of 20 mM and heating to 65 °C for 10 mins. After digestion, DNA fragments were seperated by agarose gel electrophoresis.

3.4 Agarose gel electrophoresis of digested DNA

Electrophoresis of DNA was carried out in a horizontal slab gel consisting of 0.8 % (w/v) agarose (Sigma) dissolved in 1x TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.02 M EDTA). DNA was loaded into the agarose gel in gel loading buffer (5 % (v/v) glycerol, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanol) and electrophoresis was carried out in 1x TBE buffer containing 1 µg/ml of ethidium bromide, at 70 volts. Following electrophoresis, the DNA bands were visualised on a UV transilluminator and were compared to a known DNA molecular weight ladder. The marker used was bacteriophage λ DNA (Promega) which had been digested with Hind 111 and EcoR1 restriction enzymes (λ H/E) and diluted to a concentration of 40 ng/µl. Typically 10 µl of λ H/E was run on a gel.

3.5 Purification of DNA fragments from agarose gels

Once digested with the appropriate restriction enzyme (3.3), DNA fragments were electrophoresed through a 0.8% (w/v) agarose gel (3.4) and visualised on an ultra-violet (UV) transilluminator after staining with ethidium bromide. Once the band of interest

was identified, excised using a sterile scalpel and placed in a pre-weighed Eppendorf tube, the DNA was extracted from the agarose using a QIAquick Gel Extraction Kit (QIAGEN Inc.) in accordance with the manufacturers' instructions. The weight of the gel slice was determined and 3 volumes of buffer QX1 (supplied by the manufacturer) was added per volume of gel (i.e. 300 µl of buffer per 100 mgs of gel). The gel slice was dissolved in the buffer by heating at 50 °C for 10 mins and flicking every 2-3 mins. Once the gel slice was completely dissolved, 1 gel volume of isopropanol was added and mixed. A QIAquick spin column was placed in a collection tube and the sample was loaded and centrifuged for 1 min at top speed in a microfuge. The column was washed with 0.75 mls of buffer PE containing ethanol by first leaving the column standing in PE wash buffer for 5 mins and then centrifuging for 1 min. Flow-through was discarded and the column was centrifuged again to remove any residual wash buffer. The column was then placed in a fresh microfuge tube and DNA was eluted by adding 50 µl of 10 mM Tris-HCl pH 8.5 to the center of the membrane. The column was left standing for 1 min and then centrifugated for 1 min and the collected DNA was transferred to a 500 µl Eppendorf tube for storage. 5 µl of the recovered DNA was run on another agarose gel to check recovery of the fragment before ligation.

3.6 Ligation of DNA fragments

Previously digested and gel purified DNA fragments were ligated together in approximately 1:1 molar ratios and was typically 100 ng of insert to 10-50 ng of vector DNA. Ligations were carried out in 10 µl reaction volumes which contained the appropriate dilution of ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) supplied by the manufacturer and 200 units of T4 DNA Ligase (New England BioLabs). Reactions were incubated at 16 °C for 12 hours or at 20 °C for 3 hours and were used directly to transform competent *E. coli* cells.

*tra*D36 *lac*I^{q} Δ(*lac*Z)M15 *pro*A^{+}B^{+}/ *sup*E Δ(*hsd*M- *mcr*B)5(r_K-m_K- McrB^{-}) *thi* Δ(*lac*-*pro*AB). The strain used for protein production using the pET system was BL21(DE3) (F^{-} *omp*T[*lon*] *hsd*S_B ($r_B$$^{-}$ $m_B$$^{-}$; an *E.coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene). Liquid bacterial cultures were grown in Luria Broth (LB) which contained 10 g/l bacto-tryptone(Difco), 5 g/l yeast extract (Difco), 10 mM NaCl, at pH 7.5, supplemented with the appropriate antibiotic. Solid medium contained LB supplemented with 1.5 % (w/v) agar (Difco), 10 mM $MgSO_4$ and the appropriate antibiotic.

3.8 Preparation of competent *E.coli* cells

A single bacterial colony was used to inoculate 5 mls of LB and was incubated O/N at 37 °C with shaking. 1ml of the O/N culture was used to seed 50 mls of LB in a 250 ml flask which was then incubated as before for 3 hours or until the optical density at 600 nm (OD_{600}) read 0.5. The culture was chilled for 10 mins on ice and centrifuged for 10 mins at 3,000 x *g* at 4 °C. The bacterial pellet was resuspended in 25 mls of ice-cold 50 mM $CaCl_2$. Cells were incubated on ice for 10 mins and centrifuged at 3,000 x *g* for 10 mins. The supernatant was discarded and the cells were very gently resuspended in 3.33 mls of ice cold 50 mM $CaCl_2$ and kept on ice (or in the fridge) until needed. Competence of the cells increased 10-fold after 24 hours and the cells were used for up to 48 hours post-preparation.

3.9 Transformation of competent bacterial cells

100 µl of competent *E. coli* cells (3.8) were added to a 10 µl ligation reaction (3.6) and incubated on ice for at least 1 hour. Cells were then heat shocked at 42 °C for 90 seconds and placed back on ice for 1 min. Then 1 ml of LB (without antibiotics) was added to the cells which were subsequently incubated at 37 °C for 1 hour. 10 µl, 100 µl and 500 µl aliquots of the cell culture were then plated onto 3 agar plates containing the appropriate antibiotic for selection of positive transformants. Both a positive control, to check the competence of the cells, and a negative control, to ensure the untransformed bacteria could not grow in the presence of the antibiotic, were included each time. The

added to the cells which were subsequently incubated at 37 °C for 1 hour. 10 µl, 100 µl and 500 µl aliquots of the cell culture were then plated onto 3 agar plates containing the appropriate antibiotic for selection of positive transformants. Both a positive control, to check the competence of the cells, and a negative control, to ensure the untransformed bacteria could not grow in the presence of the antibiotic, were included each time. The positive control contained 10 ng of pUC19 control DNA. The plates were incubated at 37 °C O/N and colonies were picked the following day for analysis of small scale DNA preparations (3.2). Transformation efficiencies were calculated by counting the number of transformant colonies on each control plate and was routinely found to be $1x10^7$ colonies per µg of pUC19 DNA.

3.10 Preparation of oligonucleotides

The phosphoramide method was used to generate synthetic oligonucleotides which were supplied in ammonium hydroxide. The base protecting groups were removed by heat treatment for 5 hours at 55 °C and the ammonia was subsequently removed by lyophilisation. The oligonucleotide pellet was dissolved in 200 µl sterile distilled water (dH_2O) and impurities which had not dissolved were pelleted by centrifugation at 14,000 rpm in a cold benchtop microfuge. The supernatant was decanted into a fresh tube where 20 µl of 3 M NaAc (pH5.2), 2.2 µl of 1 M $MgCl_2$ and 600 µl of 100 % ethanol were added. The DNA was then precipitated at -20 °C for 30 mins and pelleted by high speed centrifugation in a cold benchtop microfuge for a further 30 mins. The supernatant was carefully aspirated off and the pellet was dried briefly under vacuum and resuspended in 100 µl of dH_2O. The oligonucleotide concentration was determined by OD at 260 nm where an OD reading of 1 is equal to 25 µg/ml of oligonucleotide. From the concentration, the molarity of the solution could be calculated using 329 daltons as the mass of 1 base.

3.11 *In vitro* transcription reactions

Negative sense RNA transcripts were generated from *in vitro* transcription reactions for transfection and CAT rescue attempts. Typically the CAT plasmid DNA template was

linearised using an appropriate blunt-cutting restriction enzyme (3.3), phenol / chloroform extracted, ethanol precipitated, dried and resuspended at a final concentration of 1 μg/μl in d H_2O. Transcription reactions contained 1 μg of linearised DNA, T7 RNA polymerase buffer supplied by the manufacturer diluted to the required concentration (40 mM Tris-HCl pH7.9, 6mM $MgCl_2$, 2mM spermidine, 10 mM NaCl)(Promega), 0.4 mM NTPs, 4 mM DTT, 20-40 units of placental ribonuclease inhibitor RNasin® (Promega), 10-20 units of T7 DNA dependent RNA polymerase (Promega) in a total volume of 50 μl. Reactions were incubated at 37 °C for 2 hours whereupon 1 unit of RQ1 RNase-Free DNase (Promega) was added and incubated for 15 mins. After DNase treatment, 5 μl of the reaction mix was run on a 0.8% agarose gel (3.4) to estimate RNA concentrations. RNA was then snap frozen in an ethanol-dry ice bath and kept there until needed for transfection.

3.12 Preparation of total RNA from SV5 infected cells

A confluent monolayer of Vero cells were grown in a 150mm x 20mm tissue culture dish and infected with SV5 W3 strain at an m.o.i. of 1. At 48 hours post infection, the medium was aspirated off and the cells were scraped into 5 mls of PBS and gently pelleted by centrifugation at 3,000 x *g* for 2 mins. Once the supernatant was discarded, the resuspension and centrifugation was repeated twice more to wash the cells thoroughly. After the final centrifugation step, the cell pellet was resuspended in 0.5 mls of RNA lysis buffer (10 mM Tris-HCl pH7.8, 10 mM NaCl, 2mM $MgCl_2$) and 50 μl of 10% NP40. The lysed cells were transferred to an Eppendorf tube for centrifugation at high speed in a cold benchtop microfuge for 5 mins. The supernatant was decanted into a fresh tube containing 180 μl STE (100 mM NaCl, 10 mM Tris-HCl pH8.0, 1 mM EDTA) and 20 μl of 20% SDS. The cell extract was then split into 100 μl aliquots and extracted twice with phenol and twice with chloroform. 10 μl of 3M NaAc pH5.3 and 250 μl of 100% ethanol was added to each aliquot. The aliquots were mixed and stored at -20 °C until needed whereupon the RNA was pelleted by centrifugation for 15 mins at high speed in a cold benchtop microfuge. The supernatant was gently

aspirated off, the pellet was dried briefly under vacuum and resuspended in 10 µl of sterile dH_2O.

3.13 Reverse transcription of cDNA from viral RNA

The reverse transcription reaction utilised total cell RNA extracted from SV5 infected cells as outlined in 3.11. 1 µl of RNA was used in a 10 µl reaction which contained 1x Taq DNA polymerase buffer (supplied by Appligene), 400 µM of each of the dNTPs, 2.5 µM primer, 0.5 µl of RNasin® (Promega) and 200 units of Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (Gibco-BRL). Two negative controls were also included; one without RNA and one without reverse transcriptase. Each reaction was incubated at 37 °C for 30 mins and the resultant cDNA strand from the reverse transcription step was amplified by polymerase chain reaction (PCR).

3.14 Polymerase chain reaction (PCR) amplification of DNA

Typically 1-10 ng of plasmid DNA (or 10 µl of a reverse transcription reaction) was used as the template for polymerase chain reaction (PCR) amplification of a specific cDNA. The PCR reaction contained 1 µM each of a forward and reverse primer, 200 µM of each of the dNTPs, 1xTaq DNA polymerase buffer (supplied by Appligene) and 2 units of Taq DNA polymerase (Appligene) in a 100 µl total volume. The PCR reaction mix was overlaid with around 50 µl of mineral oil to prevent evaporation of the reaction components during the thermal cycling. PCR amplification was carried out in a Techne DNA thermal cycler programmed to cycle at 94 °C for 1.5 mins (DNA denaturation), 55 °C for 1.5 mins (primers anneal to template DNA), 72 °C for 2.0 mins (primer extension by Taq DNA polymerase), for 30 cycles with a 10 min extension at 72 °C at the end. After 30 cycles, the mineral oil was removed and 10 µl was loaded onto an 0.8% (w/v) agarose gel to check that the product was of the expected molecular weight. Once this had been confirmed, the PCR product was phenol / chloroform extracted, ethanol precipitated, dried and resuspended in 50 µl of dH_2O. The PCR product was now ready for restriction digestion and cloning into the appropriate vector.

3.15 Preparation of plasmid DNA for dideoxynucleotide sequencing

Large scale plasmid preps (3.1) were used in DNA sequencing reactions. The plasmid DNA first had to be denatured before starting the sequencing reaction. For each reaction, 5 µg of pure plasmid DNA ($OD_{260-280}$ = 1.65-1.85) was denatured by adding equal volumes of 0.4M NaOH / 4mM EDTA (i.e. 0.2M NaOH / 2mM EDTA final concentration) in an Eppendorf tube and incubating at 37 °C for 30 mins. Then one tenth (1/10) volume of 3M NaAc pH5.2 and 2.5 volumes of 100% ethanol were added and the DNA was precipitated by incubating at -20 °C for 15 mins. The DNA was pelleted by centrifugation at high speed in a cold benchtop microfuge for 15 mins. The supernatant was aspirated off and the pellet was washed in 70% (v/v) ethanol and dried under vacuum. The dried, denatured DNA pellet was stored at -70 °C until needed for the sequencing reactions. Typically 5 aliquots of a given plasmid were denatured simultaneously to allow more than one sequencing reaction at a time to be performed.

3.16 Dideoxynucleotide sequencing reactions

Sequencing reactions were carried out using a Sequenase ™ Version 2.0 DNA sequencing kit (United States Biochemical) which used the method of Sanger et al (1977). An aliquot of denatured plasmid DNA (3.15) was resuspended in 7 µl of dH_2O, 2 µl of Sequenase buffer (40 mM Tris-HCl pH7.5, 20 mM $MgCl_2$, 50 mM NaCl) and 1 µl of primer pre-diluted to 3 ng/µl. The primer was annealed to the DNA by heating at 65 °C for 2 mins and cooling slowly to 35 °C. The primer extension step utilised Sequenase V2 T7 DNA polymerase to incorporate [^{35}S]-dATP into the nascent DNA. To the cooled DNA, 1 µl of 0.1M DTT, 2µl of labelling mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl (5 µCi) of [^{35}S]-dATP (Amersham International) and 2 µl of Sequenase V2 T7 DNA polymerase diluted one in eight in enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) were added. The reactions were mixed and incubated at RT for 5 mins. The extension reactions were stopped by adding 3.5 µl of the labelled mix to each of four termination tubes containing 2 µl of the

whereupon 3 µl of each sample was loaded onto 7 M urea, 6% (w/v) polyacrylamide gel for electrophoresis.

3.17 Electrophoresis of sequencing reactions

Sequencing gels consisted of 1xTBE, 7M urea and 6% (w/v) acrylamide in a total volume of 75 mls. To this a few grains of bromophenol blue were added and dissolved to enable bubbles in the gel to be easily visualised while pouring. The solution was filtered through a 0.45 µm disposable filter and polymerised by the addition of 75 µl of 25% (w/v) ammonium persulphate and 75 µl of TEMED (BioRad). The gel solution was poured into a Sequi-Gen® nucleic acid sequencing cell (BioRad) which had been assembled according to the manufacturers' instructions and had been sealed at the bottom with 1% (w/v) agarose. Once poured, the comb was inserted into the top of the gel such that the flat edge of the comb created an even surface to the top of the gel when polymerised. After polymerisation, the comb was removed, the gel was submerged in 1xTBE and pre-run at 2,000 volts until it had warmed to 50 °C. Once the gel was warm, the comb was inserted such that the fine sharks teeth points of the comb rested on the gel surface. The spaces between the teeth created the wells where the samples were loaded so a tight fit was essential to prevent leakage of sample from well to well. The samples were boiled for 3 mins and 3 µl of each sample was loaded onto the gel for electrophoresis. The gel was routinely run at 2,000 volts until the bromophenol blue reached the bottom but it was sometimes necessary to run the gel for longer to analyse sequences further from the primer. Once run, the gels were transferred onto 3mm Whatman filter paper and dried at 80 °C under vacuum. Once dry the gels were put against X-ray film for autoradiography at room temperature. The DNA sequence was read from bottom to top of the gel / autoradiogram which represented sequence in the 3' to 5' orientation.

sequence was read from bottom to top of the gel / autoradiogram which represented sequence in the 3' to 5' orientation.

4. Generation of inducible cell lines expressing SV5 proteins

4.1 Transfection of BalbC cell for the generation of inducible cell lines

75 cm^2 tissue culture flasks were seeded with BalbC cells and grown to 80% confluency in GMEM+10% NBCS. The cells were then transfected with the driver fusion plasmid pMR101/tTA, and the appropriate responder plasmid(s) at a 1:10 driver fusion:responder plasmid ratio. For the cell lines inducibly expressing NP, P or V this was 200 µg of responder plasmid (pTET-βGlobinNP, pTET-P or pTET-V) and 20 µg of driver fusion plasmid (pMR101/tTA). Transfection cocktails contained DNA diluted to a total volume of 500 µl in OPTIMEM®. For each transfection mix 100 µl of liposomes was added to 400 µl of OPTIMEM® and mixed. The diluted liposomes were added to the DNA and incubated at RT for 20 mins. During this time, cell monolayers were washed in 5 mls of OPTIMEM®. Then 4 mls of OPTIMEM® was added to the DNA-lipid mixture which was mixed and added to the washed cells. Cells were incubated O/N at 37 °C under 5% CO_2 whereupon the transfection mixture was replaced with 20 mls of GMEM+10% NBCS and incubated for a further 24 hours. At 48 hours post-transfection, cells were trypsinised and resuspended in 10 mls of GMEM+10% NBCS. 35 mls of GMEM+10% NBCS supplemented with 500 µg/ml Geneticin (Gibco-BRL) and 2 µg/ml tetracycline (Sigma) was added to two 150 mm diameter tissue culture plates per transfection. Then 6 mls of transfected cells were added to the first dish and 2 mls to the second. (Two dilutions were used for isolation of well separated single transfectant colonies.) The rest of the cells were used in immunofluorescence (IF), (5.7) and western blotting (5.6) to monitor transient expression of the protein of interest. The medium was changed every 2 days on the 150

mm dishes to remove dead cell debris and allow the formation of geneticin resistant colonies.

4.2 Isolation of Geneticin resistant colonies.

Resistant colonies were harvested when easily visible to the naked eye. Each colony was scraped off the plastic with a sterile pasteur pipette in 200 µl of medium and transferred into a well of a 24 well tissue culture dish containing 1 ml of GMEM+10% NBCS supplemented with geneticin and tetracycline. Typically 48 clones per new cell line were harvested.

4.3 Screening of new cell line clones

Once the clones had grown to cover around one third of the well, they were trypsinised and resuspended in 500 µl medium. 50µl of each clone was dotted onto a well of a multispot slide and cells were allowed to settle on the slide O/N. Slides were washed three times in PBS to remove all traces of tetracycline and incubated in GMEM+10% NBCS for 48 hours for analysis of protein expression by immunofluorescence microscopy (5.7). Clones exhibiting positive fluorescence for the protein(s) of interest were then subcloned to increase the percentage of expressing cells (4.4).

4.4 Subcloning of positive clones

Clones exhibiting positive fluorescence for the protein(s) of interest usually had around 30-40% of positive cells but this was as low as 5% for some clones. The best clone from each cell line was therefore subcloned to generate colonies from single cells. The rationale was to expand a colony from a single cell which expressed the protein of interest, thus, theoretically, a subclone from each cell line would express the required protein of interest. Cells from the appropriate positive clone were trypsinised from the well of a 24 well dish and resuspended in 1 ml of medium. The cells were then counted in an Improved Neubauer counting chamber (Hawksley Cristalite) and cells were plated on three 90 mm diameter tissue culture dishes (100, 10 and 1 cell per dish) in GMEM+10% NBCS supplemented with geneticin and tetracycline. Colonies began to

remaining after the counting procedure were used to seed a 25 cm^2 tissue culture flat per clone and grown to confluency for storage in liquid nitrogen (4.6).

4.5 Ring cloning of single cell colonies

24 subclones, each derived from a single cell, were picked for screening by ring-cloning. A 1 cm diameter, sterile, metal ring was dipped in sterile vaseline and the vaseline coated base was placed around a clone without touching any neighbouring clones. Once secure, the vaseline seal prevented any leakage of medium outwards or inwards. The medium surrounding the clone was aspirated off and the subclone was trypsinised in 100 µl of trypsin and added to 400 µl of GMEM+10% NBCS supplemented with geneticin and tetracycline. 50 µl was then dotted onto a multispot slide for screening by immunofluorescence (5.7). 24 subclones per cell line were isolated in this way and expression from all positive subclones was verified by western blot analysis (5.6). Once positive subclones had been identified they were grown to confluency in 25 cm^2 tissue culture flats and frozen for storage in liquid nitrogen.

4.6 Freezing down of tissue culture cells

Cell monolayers were grown to confluency in 25 cm^2 tissue culture flasks and trypsinised. Cells were resuspended in 5 mls of ice-cold freezing mix (60% (v/v) GMEM, 30% (v/v) NBCS, 10% (v/v) DMSO) on ice and aliquotted into 1 ml amounts in cryotubes. Aliquots were frozen at -70 °C in a polystyrene box O/N and transferred into liquid nitrogen for long term storage.

5 Protein Analysis

5.1 Antibodies

The panel of monoclonal antibodies (mAbs) used in the course of this study were raised against SV5 proteins as described in Randall *et al* (1987), and are detailed at the front of the thesis. NP and P proteins were detected using specific mAbs while V was detected

of the thesis. NP and P proteins were detected using specific mAbs while V was detected using a mAb which also detected the P protein. L was detected in immunoprecipitation reactions (5.4) using an N-terminal peptide antiserum. This was raised against E-I-L-L-P-E-V-H-L-N-S-P-I-V-K-H-K peptide found in the hydrophilic N-terminal regions of SV5 and hPIV2 and was generously supplied by Dr.Y. Ito, Dept of Microbiology, Mie School of Medicine, Mie-Prefecture, Japan.

5.2 ^{35}S labelling of proteins

Cells were seeded in 6 well dishes, infected with VacT7 and transfected with the appropriate vector encoding an SV5 gene under T7 control as described elsewhere. At 17 hours post-vaccinia virus infection, cells were starved for 30 mins in methionine and glutamine free medium ((Gibco-BRL) supplemented with 1% NBCS. Cells were then labelled in 500 µl of labelling solution (50-100 µCi / ml of protein labelling (^{35}S-methionine/^{35}S-cysteine) mix (NEN), 2mM glutamine, 1% NBCS in methionine and glutamine free medium) for 2 hours at 37 °C. Cells were then washed twice in ice-cold PBS and prepared for immunoprecipitaion (5.4).

5.3 *In vitro* Transcription / Translation

In vitro transcription / translation reactions were performed using the TNT® Coupled Transcription / Translation System (Promega) according to the manufacturers' instructions. A typical 50 µl reaction contained 1 µg of template DNA, 20-40 units of RNasin®, 1 mM of Amino acid mix minus methionine, 40 µCi of [^{35}S]-methionine (Amersham), TNT® Reaction buffer, 1 µl of TNT® T7 RNA polymerase and 25 µl of TNT® Reticulocyte Lysate. The reactions were incubated at 30 °C for 120 mins and labelled polypeptides were analysed by SDS-PAGE (5.5). The gel was then dried on 3 mm Whatman filter paper at 80 °C under vacuum and exposed to X-ray film for autoradiography.

5.4 Immunoprecipitation

After ^{35}S-labelling, the cells were washed twice in ice-cold PBS and prepared following a protocol adapted from Harlow and Lane (1988). Cells from each well were scraped into 100 µl immunoprecipitation (IP) buffer (0.65M NaCl, 20 mM Tris-HCl pH7.8, 1 mM EDTA, 0.5% (v/v) NP40, 0.1% (v/v) NaAz, 0.1% (v/v) SDS) and transferred into 1.5 ml Eppendorf tubes. The cell lysates were sonicated using an ultrasonic probe and centrifuged at 14,000 rpm in a cold benchtop microfuge for 1 hour. The supernatants containing soluble antigens were decanted into fresh tubes and incubated with the appropriate mAbs for 1 hour on ice. Then 30 µl of Protein A sepharose beads (Pharmacia) was added to each tube, flicked to mix and incubated on ice for 1 hour. Beads were gently pelleted by low speed centrifugation for 5 mins on a benchtop microfuge and the supernatant discarded. The beads were washed five times in 1 ml of IP buffer. The final pellet was resuspended in 50 µl of SDS-lysis buffer (50 mM Tris-HCl pH 7.0, 2% (v/v) SDS, 5% (v/v) β-mercaptoethanol, 3% (v/v) glycerol, 0.1% (w/v) bromophenol blue), boiled for 3 mins and labelled polypeptides were separated by electrophoresis through and SDS-polyacrylamide slab gel (5.5). After electrophoresis, the gel was dried on 3 mm Whatman filter paper at 80 °C under vacuum and subsequently exposed to X-ray film at room temperature (RT).

5.5 SDS-Polyacrylamide Gel Electrophoresis

Proteins were denatured in SDS-lysis buffer (50 mM Tris-HCl pH 7.0, 2% (v/v) SDS, 5% (v/v) β-mercaptoethanol, 3% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and boiled before loading onto a polyacrylamide gel containing SDS for polypeptide separation by electrophoresis (Laemmli, 1970). Mini Protean System gel rigs (BioRad) were assembled according to the manufacturers' instructions. The resolving gel consisted of 0.375M Tris-HCl pH 8.3, 10% (v/v) acrylamide-bisacrylamide (29:1 acrylamide:bisacrylamide)(NBL Gene Sciences Ltd), 0.375% (v/v) ammonium persulphate, 0.1% (v/v) SDS and 4.5 µl of TEMED (BioRad) per 10 mls of gel solution. Once prepared, the gel solution was poured into pre-assembled rigs and covered with

0.5 mls of isopropanol until the gel had polymerised. Once the resolving gel had polymerised, the isopropanol was removed and replaced with stacking gel solution which consisted of 0.125M Tris-HCl pH 7.0, 0.1% (v/v) SDS, 3.75% (v/v) acrylamide-bisacrylamide (29:1), 0.056% (v/v) ammonium persulphate and 6 µl of TEMED per 4 mls of gel. Once poured, a 10 well plastic comb was inserted to create wells and the stacker was polymerised for 10 mins. Once polymerised,the comb was removed and the wells were rinsed in d H_2O before assembling into the electrophoresis tank. Gels were loaded and run at 150V in SDS-running buffer (0.025M Tris, 0.19M glycine, 0.1% SDS) until the bromophenol blue from the samples had reached the bottom of the gel.

5.6 Western Blotting

Once the polypeptides had been separated by SDS-PAGE (5.5), they were transferred to nitrocellulose membrane by western blot using a Trans-Blot Cell (BioRad) assembled according to the manufacturers' instructions. The transfer unit was submerged in transfer buffer (0.025M Tris, 0.19M glycine, 20% (v/v) methanol) in a gel tank containing an ice cooling chamber and run at 200 mA for 1 hour. After transfer to nitrocellulose, the polypeptides were detected using mAbs described in 5.1. Non-specific protein binding sites on the nitrocellulose filter were blocked by incubating the filter in Blotto (10% (w/v) skimmed milk powder, 0.2% (v/v) Tween20 in PBS) for 1 hour. Specific viral polypeptides were detected by incubating the filter with a dilution (1/100 or 1/1000) of the appropriate mAbs in Blotto for 30 mins and unbound mAb was removed by four 15 min washes in Blotto. The bound mAb was detected by incubating the filter in a 1/1000 dilution of anti-mouse Ig conjugated to horseradish peroxidase (Amersham International) in Blotto for 30 mins. The filter was washed by two 15 min washes in Blotto, a 15 min wash in PBS+0.2% (v/v) Tween 20 and a 15 min wash in PBS. Secondary antibody was detected in an enhanced chemiluminescence (ECL) reaction (Amersham) followed by autoradiography.

5.7 Caesium chloride (CsCl) gradients

Attempts were made to analyse the solubility of NP-P and NP-V complexes by their mobility through a CsCl gradient by density centrifugation, following the protocol of Buchholz *et al*, 1993. Four 25 cm^2 tissue culture flasks containing BalbC cells expressing SV5 NP, NP+P, NP+V or naive BalbC cells respectively, were induced for 48 hours by incubating the cells in the absence of tetracycline. Cells were harvested in 500 µl of a hypotonic buffer (10mM Tris-HCl, pH 8.0, 0.1M EDTA) and transferred to Eppendorf tubes. Cells were then vortexed and centrifugated at 10,000 rpm in a benchtop microfuge at 4 °C. Supernatants were decanted for loading onto the CsCl gradients.

20%, 30% and 40% CsCl solutions (w/w), and a 30% gycerol (w/w) solution were prepared in TNE (Tris-HCl, pH7.4, 50 mM NaCl, 2 mM EDTA). A 4 ml step gradient (1 ml of 40%, 30% then 20% CsCl followed by 1 ml of 30% glycerol) was assembled in centrifuge tubes suitable for the Beckman SW 50.1 rotor. The samples were layered onto the glycerol cushion and the tubes were centrifuged at 36, 000 x *g* at 16 °C for 16 hours.

Samples were harvested by carefully inserting a capillary tube through the gradient until it almost reached the bottom. A peristaltic pump was then used to extract seven 0.5 ml fractions. The polypeptides within each fraction were seperated on a 10% SDS-polyacrylamide slab gel and analysed by western blot (See 5.6).

5.8 SPOTS analysis

A series of C-terminal overlapping peptides covering the unique region of the P protein were generated on a cellulose membrane by GENOSYS (Cambridge, UK). This membrane was then reacted sequentially with the available P specific mAbs (Randall *et al*, 1986) to determine their specific sequence binding motif(s). First, the non-specific binding sites on the membrane were blocked by overnight incubation at 4 °C in a casein-based blocking reagent supplied by the manufacturer. (Blocking buffer was supplied as x10 concentrated and was therefore diluted 10 fold in Tris buffered saline

blocking buffer to approximately 0.1 mg / ml and incubated with the membrane at RT for 30 mins with shaking. The membrane was then washed five times in PBS containing 0.05% Tween 20 (PBS-T), for 10 mins per wash. The secondary antibody (HRP-conjugated sheep anti-mouse Ig) was then reacted with the membrane at RT with shaking. The membrane was washed five times in PBS-T as described above. Bound antibody was then detected in an ECL reaction as for western blots (See 5.6).
Each P mAb was tested individually and the membrane was regenerated before the next assay. Membrane regeneration involved incubation of the membrane in a solution of 5M urea / 1% SDS (v/v) / 0.1% (v/v) β mercaptoethanol for 30 mins at RT. The membrane was then washed in a solution of Ethanol / water / acetic acid (5:5:1 v/v/v) for 30 mins. The membrane was finally rinsed in PBS-T and stored in blocking buffer at 4 °C in preparation for the next assay.

5.9 Immunofluorescence microscopy

Cells were plated on multispot slides and grown until around 70% confluent in GMEM+10% NBCS. Slides were carefully washed twice in PBS and fixed in a PBS solution containing 5% (v/v) formaldehyde and 2% (w/v) sucrose for 10 mins. Cells were then permeabilized for 5 mins in a PBS solution containing 10% (w/v) sucrose, 0.1% (v/v) NaAz and 0.5% (v/v) NP40. The slides were washed three times in PBS and incubated with the appropriate mAb - NP was detected with SV5 NP-d conjugated to the fluor Texas Red, both the P and V proteins were detected with SV5 P-k conjugated to fluorescein isothiocyanate (FITC). Both antibodies contained DAPI (1.4) at 10 µg/ml as an indication of cell density and also monitored mycoplasma contamination. 50 µl of a 1/100 dilution of the mAb was added per well to the multispot slide and incubated for 1 hour at 37 °C in a humid atmosphere. If two proteins expressed simultaneously were to be detected by double staining, the mAbs were mixed before incubation with the cells. When the incubations were complete, the slides were washed three times in PBS and fixed for a further 5 mins. The slides were washed again in PBS and air dried briefly before adding 2-3 µl Citifuor mounting fluid (Citifluor Ltd) per well, covering

briefly before adding 2-3 µl Citifuor mounting fluid (Citifluor Ltd) per well, covering the slide with a clean coverslip and examined using the immonofluorescence microscope.

5.10 Chloramphenicol Acetyl-Transferase (CAT) Assays

This CAT assay method was adapted from that of Gorman *et al*, (1982). Transfected cell monolayers in 6 well dishes were washed with 3 mls of ice-cold PBS and scraped into 1 ml of harvesting buffer (40 mM Tris-HCl pH7.4, 1mM EDTA, 150mM NaCl). Cells were transferred into 1.5 ml Eppendorf tubes and pelleted by centrifugation at 1,500 rpm in a cold benchtop microfuge for 5 mins. The supernatants were poured off and pellets were resuspended in 50 µl of 0.25M Tris-HCl pH 7.5 by vortexing briefly. The cells were lysed by repeated freeze / thaw cycles in an ethanol / dry ice bath and 37 °C water bath. Cell debris was pelleted by high speed centrifugation in a benchtop microfuge at 4 °C for 5 mins. During this time, a bulk CAT reaction mix was prepared. Each reaction contained 70 µl Tris-HCl pH 7.5, 24 µl dH_2O, 2µl of 40 mM Acetyl Coenzyme A and 4µl (0.1 µCi) of ^{14}C-chloramphenicol (Amersham). Once the bulk mix was prepared, it was aliquotted in 100 µl amounts into a set of tubes on ice. The supernatant from the lysed cells was added to the reaction mix and incubated in a 37 °C water bath for 2-24 hours. After incubation, the reactions were extracted with 1 ml of ethyl acetate by vortexing and spinning at 14,000 rpm in a cold benchtop microfuge for 10 mins. The top layer containing chloramphenicol and the acetylated products was decanted into a fresh tube. The lid of each tube was pierced and the samples were dried under vacuum for 1 hour at 37 °C. Once dry the samples were resuspended in 20 µl ethyl acetate and dotted onto a silica gel plate for thin layer chromatography (TLC). The plate was then placed in a TLC tank with a saturated atmosphere 95 chloroform : 5 methanol until the solvent front was approximately 1 cm from the top of the plate. The plates were removed from the tank, air dried and put against X-ray film for autoradiography at RT.

5.11 β-Galactosidase Assays

To determine the transfection efficiency of a particular cell line or a new batch of home-made cationic liposones (2.3.2), cells were seeded at $1x10^6$ cells per well in 6 well tissue culture dishes. When the cells were around 80% confluent, they were transfected with a known amount of pPEβGal plasmid DNA which contained the bacterial *lac*Z gene encoding the β-Galactosidase enzyme under the control of the human cytomegalovirus (hCMV) immediate early promoter. The cells which had been transfected, expressed β-Galactosidase and therefore stained blue in the presence of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-galactopyranisode). By counting the number of blue cells the percentage of transfected cells was determined. 36 hours post transfection the cells were washed in PBS and fixed for 5 mins at room temperature in 1 ml of a PBS solution containing 2% (v/v) formaldehyde and 0.2% (v/v) gluteraldehyde. The fixing solution was aspirated off and replaced with 1 ml of a staining solution (PBS containing 5 mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM $MgCl_2$ and 0.5 mg/ml of X-Gal in DMF) and incubated in the dark until the colour appeared. Typically the blue colour was visible to the naked eye after 2 hours. The reaction was stopped by washing the monolayers in PBS and the percentage of transfected cells was determined by light microscopy.

5.12 Preparation of an Infected Cell Extract (ICE)

During the preparation of SV5 W3 virus stock (1.2.1), 4 confluent roller bottles of Vero cells were infected with virus. Once the medium containing the virus had been harvested, the infected cells were also harvested to prepare an infected cell extract (ICE) for western blot analysis. The migration of the viral polypeptides in a polyacrylamide gel acted as a control for the migration of those from recombinant proteins. The cells were harvested by the addition of 10 mls of ice-cold PBS to one roller bottle whereupon around 100 sterile glass beads were added and the bottle was shaken to dislodge the monolayer. Once dislodged, the PBS containing the cells was decanted into a 50 ml screw-capped tube and the cells were pelleted by centrifugation at 4,000 x *g* for 5 mins.

The supernatant was carefully poured off and the cell pellet was resuspended in 5 mls of SDS-lysis buffer (5.5) and sonicated with an ultrasonic probe for 30 secs at high power three times. The cell lysate was aliquotted in 1 ml amounts and stored at -20 °C. Before loading onto an SDS-polyacrylamide slab gel for electrophoresis and western blotting, the ICE was diluted 1/20 in SDS-lysis buffer, boiled and 10 µl was typically loaded onto a gel.

5.13 Capture Assays

5.13.1 Initial Capture Assay Protocol

To look at viral protein:protein interactions, a novel solid phase panning assay was developed by Dr.R.E. Randall. This involved flooding a 96 well microtitre plate with a 0.2% (v/v) suspension of fixed and killed *Staphylococcus aureus* Cowan Strain A in PBS for at least 4 hours at 4 °C. The unbound material was removed by washing three times in PBS and the remaining *S. aureus* was cross-linked to the plastic by incubating in fixing solution (5.6) for 10 mins at RT. Once fixed, the plate was washed again whereupon 50 µl of a 1/100 dilution (in PBS) of an appropriate mAb was added to each well and incubated for 2-3 hours at 4 °C with rocking. Unbound mAb was removed by washing three times in PBS and non-specific protein binding sites were blocked by flooding the plate with 10% (w/v) bovine serum albumin (in PBS) or Blotto (5.6) and incubating for 1 hour at 4 °C. The plate was again washed in PBS whereupon 100 µl of P or V containing cell extract (See below) per well was added and incubated for 1-2 hours on a rocker at 4 °C. Unbound material was removed by washing the plate four times in PBS. To examine the binding of NP to captured P or V proteins, 100 µl of NP extract was added to the appropriate wells and bound for 1-2 hours at 4 °C on a rocker. Any unbound material was again removed by washing in PBS. Bound polypeptides were harvested by adding 30 µl of SDS-lysis buffer (5.5) per well. The plate was boiled for 5 mins and samples were loaded onto a 10% SDS-PAGE (5.5) for western blotting (5.6).

5.13.2 Amended Capture Assay Protocol

The original capture assay protocol described formaldehyde cross-linking of the *S.aureus* to the microtitre plate followed by the addition of a mAb of interest. When the proteins on each well were analysed by western blot, the heavy and light chains of the immobilising antibody were visualised and sometimes obscured the the band of interest. To circumvent this problem, formaldehyde was used to cross-link the immobilising mAb to the *S.aureus* on the plate.

A 96 well plate was flooded and incubated with *S.aureus* as described above. The plate was washed and then 50 µl of a 1/100 dilution (in PBS) of an appropriate mAb was added per well. The plate was incubated at 4 °C for 2-3 hours on a rocker. Unbound mAb was removed by washing the plate in PBS whereupon the mAb was cross-linked to *S.aureus* by flooding the plate with fixing solution (5.7) for at least 3 hours at RT. Once fixed, the plate was washed in PBS and non-specific protein binding sites were blocked by the addition of Blotto (5.6) for 1 hour at RT. The plate was again washed three times in PBS before adding cell extracts as described in 5.11.1.

5.14 Preparation of cell extracts for capture assay

5.14.1 Preparation of extracts from cell lines inducibly expressing P or V proteins

BalbC cells which expressed P of V proteins were seeded onto five 150 mm diameter tissue culture dishes per cell line and grown to 60% confluency in GMEM+10% NBCS supplemented with geneticin and tetracycline (4.2). The P or V proteins were induced for 48 hours in the absence of tetracycline. Cells were harvested by scraping into 5 mls of ice-cold PBS per plate and pelleted by centrifugation at 4,000 x *g* for 5 mins in 50 ml screw-capped tubes. The supernatant was carefully poured off and the cell pellet was resuspended in 10 mls of PBS. Cells were stored at -70 °C in 1 ml aliquots until required. When required, cell extracts were thawed, sonicated for 30 secs and cell debris was pelleted by centrifugation at 14,000 rpm in a benchtop microfuge at 4 °C for 15

15 mins. The supernatant was decanted into a fresh tube and 100 µl of the P or V extract was used per well on a capture assay plate.

5.14.2 Preparation of extract from cell line inducibly expressing NP protein

BalbC cells were seeded, induced for 48 hours and harvested as described in 5.12.1. Cell extracts were made by sonicationg the thawed NP aliquot for 30 secs and spinning at 6,500 rpm on a benchtop microfuge for 10 mins. The supernatant was decanted into a fresh tube and called "Low Speed Supernatant" (LSS). The LSS was further fractionated by centrifugation at 400,000 x *g* in a Beckman benchtop ultracentrifuge. The supernatant was decanted and called "High Speed Supernatant" (HSS), and the pellet was resuspended in its original volume of PBS by sonication and was called "High Speed Pellet" (HSP). 100 µl of each extract was used per well on a capture assay plate.

5.14.3 Preparation of P-deletion mutant extracts from VacT7 infected cells

A 90 mm diameter tissue culture dish per construct was seeded with 293 cells and grown in GMEM+10% FCS until 80% confluent. The cells were infected with VacT7 at a m.o.i. of 1 (1.3.3) and at 1 hour post-infection, were transfected (2.3.2) with 10 µg of pTM-P or the deletion mutant derivatives. Cells were harvested at 18 hours post-infection by washing the monolayers in PBS and scraping into 10 mls of PBS per plate. The cells were gently pelleted by centrifugation at 4,000 x *g* for 5 mins and the pellets were resuspended in 1 ml of PBS. Cells were aliquotted in 100 µl amounts and stored at -70 °C until needed. When required, the aliquots were thawed, sonicated and centrifuged at 14,000 rpm in a cold benchtop microfuge for 15 mins. The supernatants were decanted and diluted 1/100 in PBS. 100 µl per well was added to to capture assay plates.

5.14.4 Preparation of P extract from bacterial cells

The pET (plasmid for Expression by T7 polymerase) vector expression system, first developed by Studier and Moffat (1986), is one of the most effective ways of expressing high levels of protein in *E.coli*. Here pET11c was used as the vector into which both the P and V open reading frames were cloned. pET11c contains the *lacI* gene encoding the Lac repressor which prevented expression of T7 RNA polymerase encoded by a λ prophage in the bacterial genome. (Bacterial strain (3.7) used was BL21(DE3)). Expression of T7 RNA polymerase was induced (or de-repressed) by the addition of isopropyl-β-D-thiogalactoside (IPTG) which inactivated the Lac repressor. The T7-RNA polymerase, in turn, bound to the T7 promoter and induced expression of the P or V proteins. Bacterial cells containing the pET-P plasmid were grown O/N from a single bacterial colony in 5 mls of LB (3.7) supplemented with 100 μg/ml of amplicillin. The O/N culture was used to seed 50 mls LB+Ampicillin (Amp) at a 1/100 dilution and the culture was grown until the OD_{600} was equal to 0.3. P protein was induced for 3 hours by the addition of IPTG to a final concentration of 1 mM. Cells were pelleted by centrifugation in 50 ml screw-capped tubes at 4,000 x *g* for 15 mins. The cell pellet was resuspended in 5 mls of PBS and aliquotted into 500 μl amounts to be stored at -70 °C until needed.

When needed, an aliquot was thawed and the cells lysed by the addition of 100 μg/ml lysozyme and incubated at RT for 5 mins. Then 500 μl of IP buffer (5.3) without SDS was added and lysate was sonicated at full power for 30 secs. Cell debris was pelleted by centrifugation at 400,000 x *g* in a benchtop ultracentrifuge (Beckman) at 4 °C for 30 mins. The supernatant was decanted into a fresh tube, diluted 1/1000 in PBS and 100 μl was used per well on capture assay plates.

5.14.5 Preparation of V extract from bacterial cells.

Supernatant from bacterial cells expressing V protein were contaminated by a significant amount of P, which had to be removed before carrying out the capture assay. The V extract was prepared as described in 5.12.3 and the clarified supernatant was again diluted 1/1000 in PBS. To remove the contaminating P protein, the diluted extract

was incubated with 500 µl of P specific mAb (SV5 P-e) bound to *S.aureus.* (1ml of a 10% suspension of fixed and killed *S.aureus* Cowan strain A was incubated with 20 mls of hybridoma cell tissue culture fluid containing expressed SV5 P-e for 1 hour on a tumbler. *S.aureus*-mAb complexes were pelleted by centrifugation at 4,000 x *g* for 5 mins and pellet was gently resuspended in 20 mls of PBS. *S.aureus*-mAb complexes were pelleted again and resuspended in 500 µl of PBS and added to diluted V extract) for 1 hour on a tubler at 4 °C. The *S.aureus*-mAb-P complexes were pelleted by centrifugation at 4,000 x *g* for 15 mins at 4 °C and the supernatant was carefully decanted into a fresh tube. 100 µl of this V extract was added per well of a capture assay plate.

Chapter 3 : Results

The 'Results' chapter has been broken down into 3 main sections with appropriate introductions to each section. The first documents early attempts made to characterise conditions which supported the transcription and replication of a synthetic negative sense RNA mini-genome using reverse genetics techniques. The rationale for this approach is explained and the generation of the necessary components for the reverse genetics system described in detail. Section 2 utilises some of the clones constructed in Section 1 for the generation, isolation and characterisation of BalbC cell lines which inducibly express SV5 proteins. The cell lines had a dual purpose; to supply SV5 proteins *in trans* for the rescue of synthetic mini-genomes (from Section 1) and to examine viral protein:protein interactions taking place within the replication complex. Immunofluorescence data are presented from these cell lines which suggest direct interactions between NP and both the P and V proteins. These interactions are examined more closely in Section 3 using a solid phase panning assay (Capture Assay) developed for this purpose and suggest possible roles for the protein interactions during virus replication.

Appendix 1

The following constructs were made by Mr. B.L. Precious in this laboratory and used by me in the experiments described in this thesis.

1) Reverse genetics experiments	:-	pUCSV5TKCAT
2) Expression of SV5 proteins	:-	pGEM-P, pGEM-V
3) Generation of inducible cell lines	:-	pMR110/tTA, pTET-P, pTET-V
4) Bacterial expression of P and V	:-	pET-P, pET-V

The inducible cell lines expressing NP, P and V, NP+P and NP+V were isolated by Mr. D. Young in this laborotory and used by me in the experiments described in this thesis. (Note that a second, more stable NP+V cell line was isolated by me, as described herein.)

1 Reverse Genetics of SV5

This section documents early attempts made to develop a defined system for the study of transcription / replication mechanisms of the *Rubulavirus*, SV5, using reverse genetics techniques. This work was carried out as part of an ongoing project started in 1991 by Mr. B. L. Precious in this lab and continued by me, in collaboration with Mr. Precious. Preceeding my arrival in the lab in 1992, Mr. Precious had constructed a cDNA clone encoding a genomic sense RNA analogue under the control of the bacteriophage T7 promoter. This clone was used by me in the rescue experiments presented in this section, and for completeness, details on the construction of this initial clone are included.

As discussed in the introduction, (Chapter 1, Section 2), viral RNA can only function as a template for transcription and / or replication when in a correctly assembled nucleocapsid structure. Therefore, five different approaches were taken which aimed to encapsidate our synthetic RNA transcript into a form recognisable to the viral polymerase complex. This first involved intracellular generation of the genome analogue in SV5 infected cells, where the encapsidation / replication proteins were supplied by the SV5 helper virus. In the second approach, the *in vitro* generated genome analogue was transfected into cells containing SV5 helper virus. In the next approach, the *in vitro* generated genome analogue was transfected into cells expressing plasmid-encoded proteins for encapsidation and transcription. The fourth approach involved the *in vitro* generation and subsequent transfection of a synthetic copyback defective interfering (DI; pan-handle) structure, into cells expressing the viral plasmid-encoded proteins. The fifth, and last, approach involved transfecting *in vitro* synthesised genome analogues into cell lines induced to express SV5 proteins (The latter is included in Section 2).

The results presented here demonstrate the problems faced in attempting to rescue mimi-genomes by supplying the proteins necessary for encapsidation, transcription and replication from SV5 helper virus or *in trans* from cDNA clones.

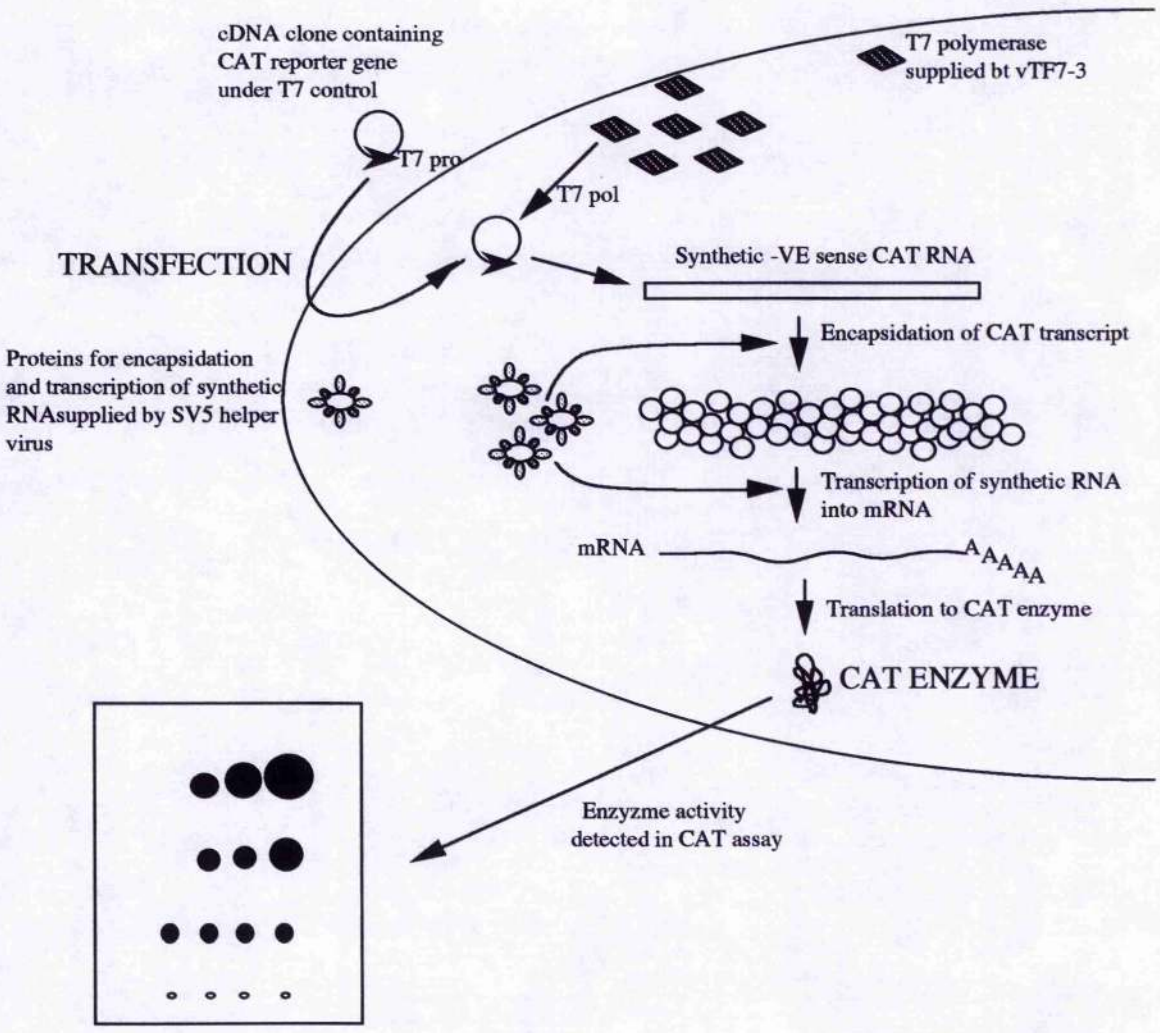


Fig.12:Transfection strategy for CAT rescue

Overview of transfection strategy for rescue of synthetic CAT transcripts from cDNA clone. Transfected plasmid DNA contains the SV5 leader and trailer sequences flanking the CAT reporter gene under the control of the T7 promoter. T7 polymerase supplied by recombinant vaccinia virus (vTF7-3) transcribes the synthetic negative sense CAT RNA. Proteins necessary for encapsidation and transcription of the synthetic transcript are supplied by SV5. The resultant mRNA is translated into CAT enzyme, the activity of which is detected by CAT assay.

1.1 Overview of mini-genome rescue system

1.1.1 Transfection strategy for CAT rescue system

The original strategy of amplification of a synthetic RNA was adapted from that of Luytjes *et al* (1989) where they expressed recombinant RNA from plasmid DNA. They replaced the coding sequence of the influenza A virus NS gene with the bacterial chloramphenicol acetyl-transferase (CAT) gene and genomic sense transcripts were generated which contained authentic terminal sequences from the influenza virus segment, flanking the CAT reporter gene. This transcript was encapsidated *in vitro* by purified virus NP and polymerase proteins (PA, PB1 and PB2) and transfected into influenza virus infected cells. In the presence of the helper virus, the recombinant RNA was amplified, expressed and packaged into virus particles which could be passaged several times. This strategy was adapted for the SV5 system as shown in Fig.12.
A plasmid was constructed which contained the SV5 leader and trailer sequences flanking 2 reporter genes all under the control of the bacteriophage T7 promoter (See Section 1.1.2 for details on construction). The reporter genes, thymidine kinase (TK) from herpes simplex virus and CAT, were separated by the intergenic region between the SV5 NP and P/V genes. It was hoped that analysis of the role(s) played by the intergenic regions in viral transcription / replication would be possible by comparing the relative activities of the two reporter genes. A recombinant vaccinia virus (vTF7-3), (Fuerst *et al*, 1986), was used to supply T7 DNA-dependent RNA polymerase which bound to the T7 promoter and generated a negative sense RNA transcript. The synthetic RNA transcript aimed to mimic the SV5 genome allowing encapsidation by SV5 proteins into a synthetic nucleocapsid structure. It was hoped that the synthetic nucleocapsid would be recognised by the viral RNA-dependent RNA polymerase complex leading to synthesis of reporter gene mRNA. Once translated, CAT or TK enzyme activity could be detected in biochemical assays. (Note that the BHK cells used in these experiments had been treated with the thymidine analog 2'-bromo-deoxyuridine (BDU), (Sigma), to generate a TK-deficient cell line.

1.1.2 Construction of a cDNA clone for CAT rescue

The rationale for the construction of the double reporter gene construct is outlined above and depicted in Fig.13 which shows the relationship between the SV5 genome and the subsequent plasmid. As mentioned previously, it was constructed and generously provided by B.L. Precious from this lab. The NP and P/V genes of SV5 were replaced by the TK and CAT reporter genes respectively, by a series of PCR reactions and cloning steps in the pUC19 cloning vector. The SV5 leader, trailer and intergenic sequences were amplified from viral RNA by reverse transcription / polymerase chain reaction (RT/PCR) amplification and verified by dideoxy-nucleotide sequencing (See Materials and Methods, Chapter 2, Section 3.13 and 3.14 for details on RT/PCR and Sections 3.15-3.17 for DNA sequencing). The CAT gene and T7 promoter were also PCR amplified but the TK gene was subcloned directly into pUC19 from pX1, a plasmid containing HSV1 macroplaque TK. (pX1 was kindly supplied by Dr. C. Preston, MRC Virology Unit, Church Street, Glasgow, UK). The resultant cDNA construct, pUCSV5TKCAT, gave rise to a negative sense RNA containing the SV5 leader and trailer sequences flanking the TK and CAT genes which were separated by the NP-P/V intergenic region, all under the control of the T7 promoter. pUCSV5TKCAT was the initial clone used in the CAT rescue experiments and the negative sense RNA transcript obeyed the rule of six (Calain and Roux, 1993; Chapter 1, Section 2.2.2).

1.1.3 Comparison of transfection methods by CAT assay

Before embarking on the CAT rescue experiments, a reliable method for DNA delivery had to be chosen. The efficiency of DNA delivery into Baby Hamster Kidney (BHK) cells was examined using three transfection methods, calcium phosphate, DEAE-Dextran and lipofection, as detailed in Material and Methods (Chapter 2, Section 2).

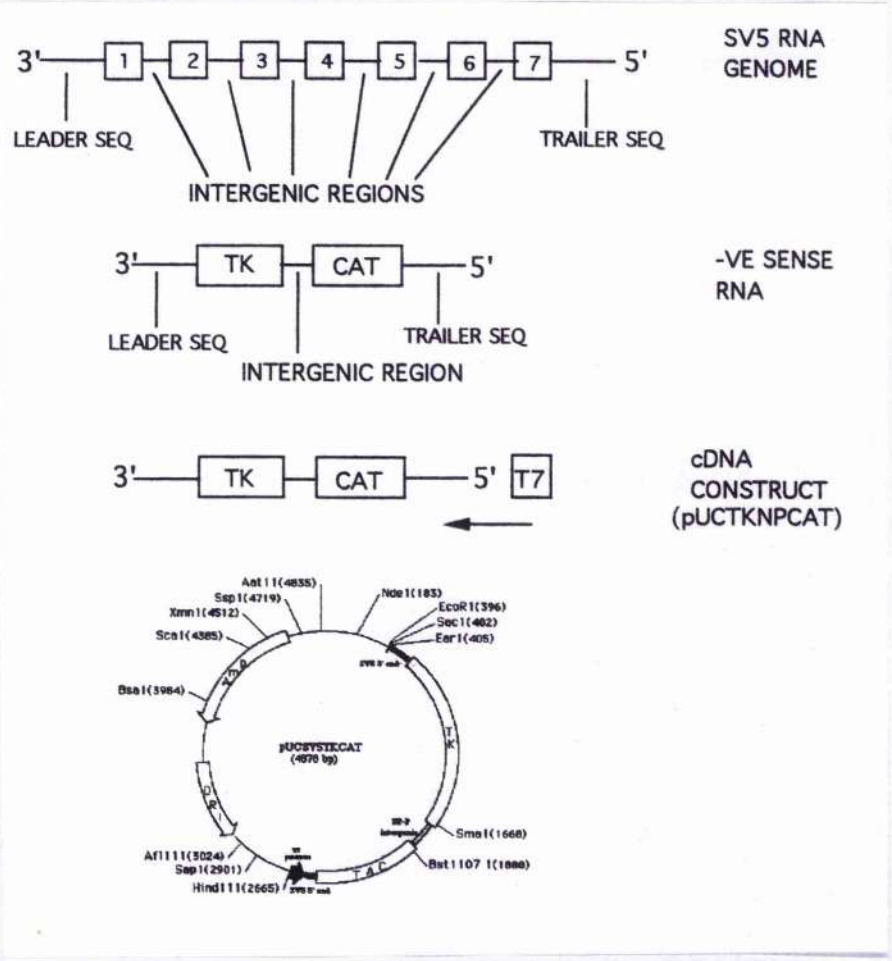


Fig. 13 : Construction of pUCSV5TKCAT

Diagrammatic representation of the SV5 genome where the NP and P/V genes are represented as genes 1 and 2 respectively. The NP and P/V genes were replaced by the reporter genes thymidine kinase (TK) and chloramphenicol acetyl-transferase (CAT) respectively, to generate pUCSV5TKCAT which was subsequently used in CAT rescue experiments.

The transfection efficiency using each method was determined by introducing pSV2CAT plasmid DNA encoding the CAT reporter gene under the control of the SV40 early promoter region (Gorman *et al*, 1982) into the cells and assaying for CAT expression. CAT enzyme inactivates chloramphenicol by the formation of mono- and diacetylated derivatives (Shaw, 1967) which can be easily separated by silica gel thin layer chromatography (Cohen *et al*, 1980). Fig.14 demonstrated that little or no difference was found between the transfection methods at the 100 ng and 1 μg level of input DNA. However, with calcium phosphate mediated transfection using HBS, CAT activity could be detected from 10 ng of input DNA and so this was deemed to be the most efficient method for DNA delivery (Compare Lane 5 with Lanes 2, 8, 11 and 14). The effect of transfection upon cell morphology was also noted, as future experiments were to involve transfecting virus infected cells and so the 'mildest', or 'least toxic' method was required. Although calcium phosphate mediated transfection using HBS was the most efficient transfection method for BHK cells, a cytopathic effect was observed. (Around 1% of the cells died and around 20% of the surviving cells had a round morphology.) This effect was seen with all the transfection methods except lipofection. Based on this observation, lipofection was used as the transfection method in future experiments since it gave a comparable level of transfection efficiency to the other methods tested but was much less toxic to the cells.

1.1.4 Monitoring transfection efficiencies by β-Galactosidase assay

Lipofection was the transfection method of choice but the commercially available Lipofectin ™ (Gibco-BRL) proved to be prohibitively expensive. Cationic liposomes, equivalent to Gibco-BRL TransfectACE ™, were subsequently prepared in the lab by the method of Rose *et al*, 1991 and used in all transfection experiments (See Chapter 2, Section 2.3.1 for liposome preparation). Due to the potential liposome batch variability, the transfection efficiency of each new batch was tested by β-Galactosidase assay (See Chapter 2, Section 5.11 for details).

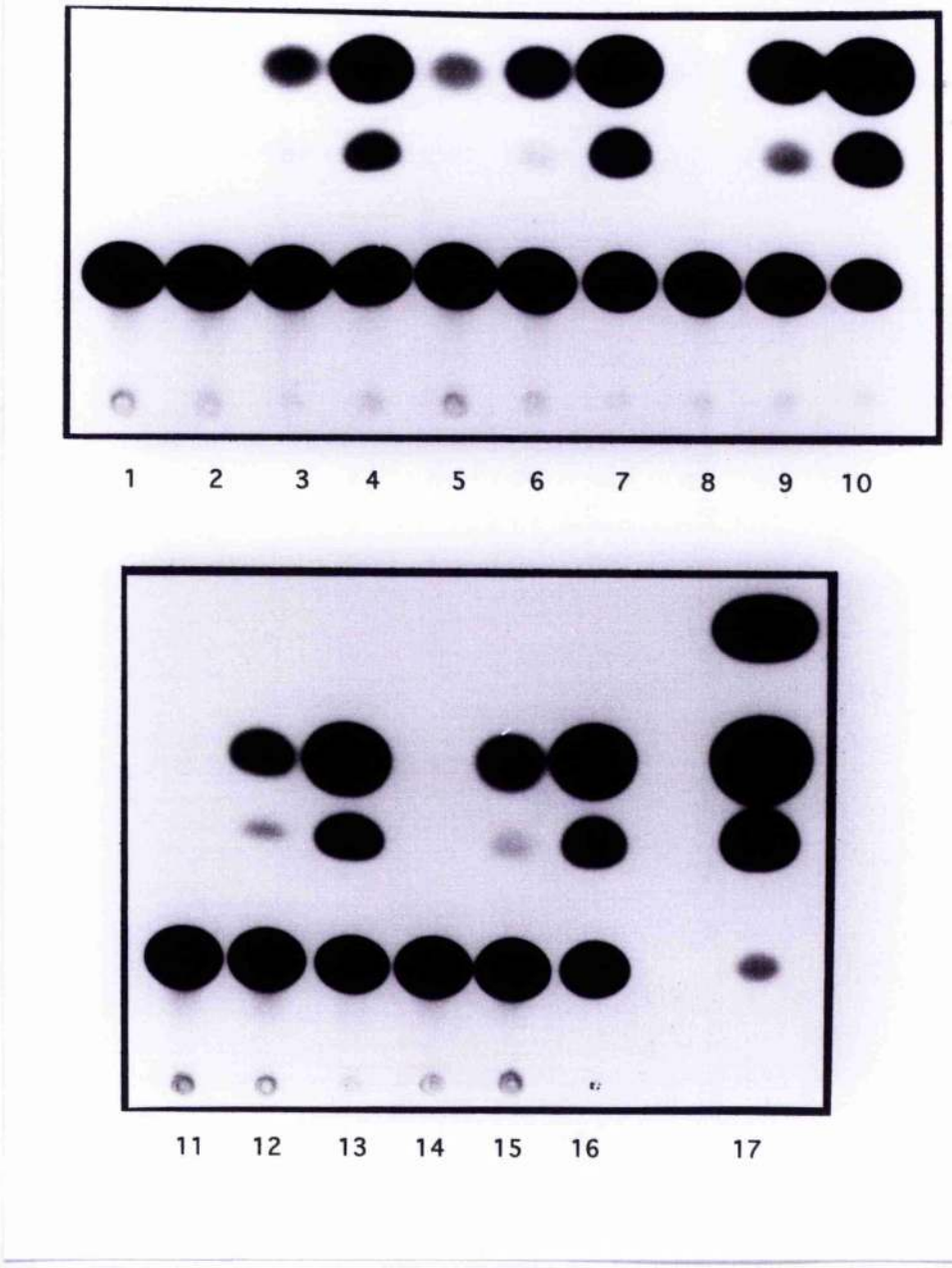


Fig.14: Comparison of transfection methods by CAT assay

Different transfection methods were used to determine which gave the highest transfection efficiency for BHK cells as judged by CAT assay. 10 ng, 100 ng and 1 μg of pSV2CAT was transfected using BBS (Lanes 2, 3 and 4 respectively); HBS (Lanes 5, 6 and 7); Lipofectin (Lanes 8, 9 and 10); Old DEAE-Dextran (Lanes 11, 12 and 13) and New DEAE-Dextran (Lanes 14, 15 and 16). A 'No DNA' control was in Lane 1 illustrating the migration of ^{14}C-chloramphenicol. Lane 17 (control) contained 5 units of CAT enzyme illustrating the acetylated forms of ^{14}C-chloramphenicol. These are (in order of increasing mobility) 1-acetate chloramphenicol, 3-acetate chloramphenicol and 1,3-diacetate chloramphenicol. Each of the transfection methods tested contained readily detectable amounts of CAT activity at the 100 ng and 1 μg level of pSV2CAT input DNA. Only HBS-mediated transfection gave detectable amounts of CAT activity from 10 ng of input DNA.

Fig.15 shows an experiment where pPEβGal, a plasmid containing the β-galactosidase gene under the control of the human cytomegalovirus (hCMV) immediate early promoter, was transfected into 293 cells with the cationic liposomes. Cells were then fixed and stained at 36 hours post-transfection. The cells which had been transfected, expressed active β-galactosidase and therefore stained blue in the presence of the chromogenic substrate X-Gal. By counting the number of blue cells, a percentage of transfected cells was determined and was typically found to be around 20% when using 293 cells. When comparing the transfection efficiency between cell types, BHK and BalbC cells were found to have a 40-fold lower transfection efficiency than 293 cells. This observation became important in future experiments.

1.2 Rescue of mini-genomes by SV5 helper virus

1.2.1 VacT7 derived CAT activity from input pUCSV5TKCAT

The strategy described in 1.1.1 and depicted in Fig.12 was used to rescue SV5-TK-CAT mRNA transcripts. It was known that an exact 3' genomic RNA end was necessary for encapsidation of a synthetic transcript (e.g. Park *et al*, 1991; Pattnaik *et al*, 1992), therefore pUCSV5TKCAT DNA was linearised by digestion with Ear1 restriction enzyme. Increasing amounts of the linearised DNA were subsequently transfected into BHK cells which had been infected with vTF7-3 (VacT7) to generate a negative sense RNA transcript (from the T7 transcription reaction) with an exact 3' end of the SV5 genome. These cells were also infected with SV5 to supply the proteins necessary for transcription and replication of the mini-genome.

No CAT activity was detectable in the test lanes containing linearised pUCSV5TKCAT (Fig.16 Lanes 1-3). One reason which could account for this was found in a subsequent experiment which showed linearised plasmid DNA had very low transfection efficiency compared to uncut (circular) plasmid. (The transfection efficiency of BHK

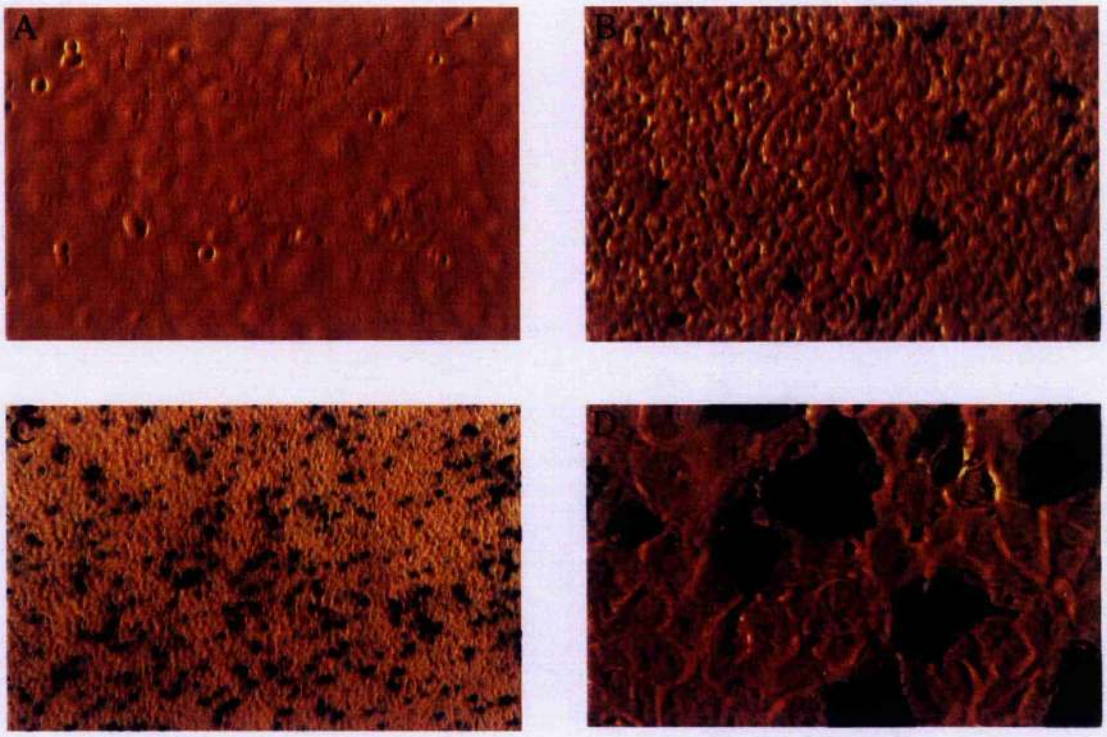


Fig.15 : Transfection efficiency by B-Galactosidase assay

B-Galactosidase assays were used to monitor transfection efficiency of 293 cells using each new batch of liposomes. Increasing amounts (0, 10ng and 100 ng) pf pPEBGal plasmid DNA was transfected into 293 cells. At 36 hours post-transfction the cells were fixed in a PBS solution containin 2% (v/v) formaldehyde and 0.2% (v/v) gluteraldehyde for 5 mins. The cells which had been transfected, exhibited B-Galactosidase activity and therefore stained blue in the presence of the chromogenic substrate X-Gal. Panel A shows a negative control where no DNA had been transfected. 10 ng and 100 ng of DNA were added to Panels B and C respectively. Panels D is a x40 magnification of the cells transfected with 100 ng of DNA. (Panels A, B and C were x20 magnification).

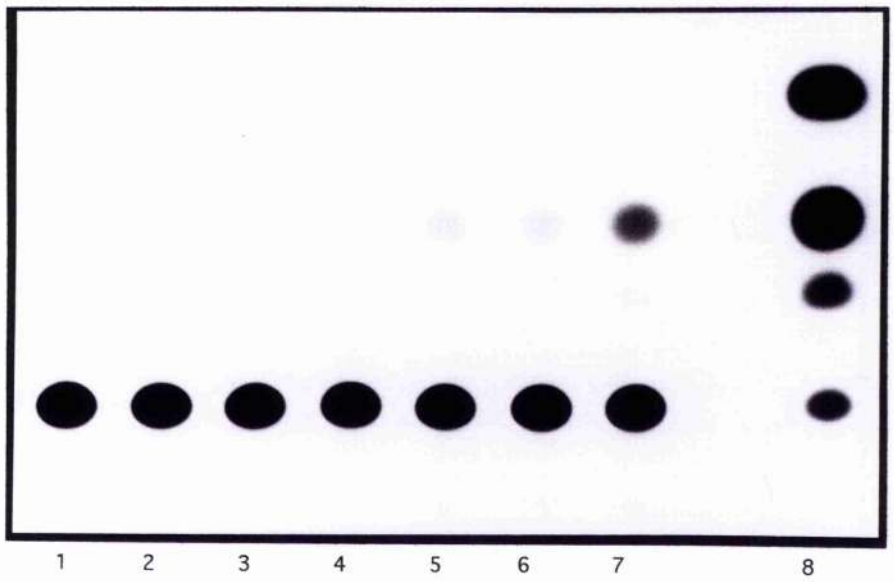


Fig.16:Transfection of BHK cells with pUCSV5TKCAT after infection with VacT7 and SV5.

BHK cells were transfected with 500 ng, 1 μg and 2 μg of linearised pUCSV5TKCAT after infection by both VacT7 and SV5 (Lanes 1, 2 and 3 respectively). Lane 4 contains cells tranfected with 2 μg of linearised pUCSV5TKCAT after infection with VacT7 alone. Cells were transfected with 2 μg circular (uncut) pUCSV5TKCAT after infection with SV5 and VacT7 (Lane 5) or VacT7 alone (Lane 6). Lane 7 contains an extract of cells which were transfected with 100 ng of pSV2CAT control DNA and Lane 8 contained 5 units of CAT enzyme control.

cells using circular (uncut) plasmid DNA was found to be around 1%, estimated by β-Galactosidase staining, but this fell to less than 0.1% when the plasmid was linearised. (Data not shown). To increase the percentage of cells being transfected, circular (uncut) plasmid was used.

The necessity of the exact SV5 3' end of the synthetic RNA was examined by transfecting 2 μg circular (uncut) plasmid DNA into cells infected with both SV5 and VacT7 (Lane 5) or VacT7 alone (Lane 6). If the encapsidation process did not require an exact 3' end then encapsidation of the transcript would begin elsewhere resulting in a viable synthetic nucleocapsid, recognisable to the viral polymerase and could therefore give rise to CAT mRNA and CAT enzyme. CAT activity was detected at a very low level in the cell extracts in Lanes 5 and 6. This was a curious finding as Lane 6 had been designed as a negative control due to the absence of SV5 helper virus. Without helper virus, the synthetic transcript generated by VacT7 could not have been encapsidated by SV5 proteins to form a synthetic nucleocapsid which, in turn, could not have been transcribed by SV5 RNA-dependent RNA polymerase to CAT mRNA for translation. Since this low level CAT activity was found when pUCSV5TKCAT was transfected into VacT7 infected cells, with or without SV5, one explanation was that the vaccinia virus itself was transcribing CAT mRNA directly from the DNA template. Although an unexpected problem, it did demonstrate that pUCSV5TKCAT had the capability of giving rise to active CAT enzyme.

1.2.2 Construction of pUCSV5CAT

To simplify the system and increase the possibility of synthetic negative (-VE) sense CAT RNA being encapsidated and transcribed into CAT mRNA, a smaller construct was made. It was anticipated that once the system was established using the smaller CAT construct, the double reporter construct could again be used to analyse the role of the intergenic regions in modulating viral transcription.

Fig.17 shows a schematic diagram of the construction of pUCSV5CAT from pUCSV5TKCAT. A fragment containing the TK gene, SV5 leader sequence (3' end),

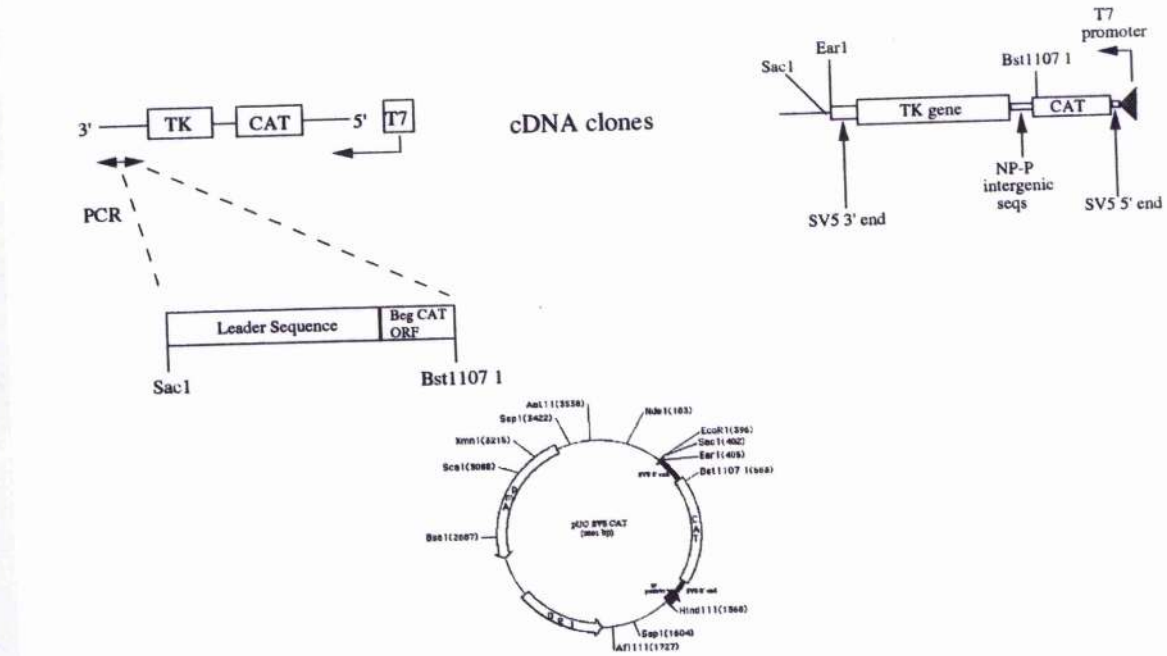


Fig.17:Construction of pUCSV5CAT

To shorten the synthetic transcript being used in transfections and CAT assays, pUCSV5TKCAT was modified to pUCSV5CAT. The TK gene, flanked by SV5 3' leader region and NP-P intergenic region, was excised by digesting with Sac1 and Bst1107 1 restriction enzymes. Bst1107 cut between bases 23 and 24 of CAT ORF. The SV5 3' leader region was replaced by PCR amplification from pUCSV5TKCAT in which the forward primer included the first 64 bases of the 3' leader sequence and the reverse primer included the first 34 bases of CAT ORF. The PCR product was also digested with Sac1 and Bst1107 1 and ligated to the digested vector to create pUCSV5CAT.

NP-P intergenic region and the first 34 bases of the CAT ORF was excised from pUCSV5TKCAT by digestion with Sac1 and Bst 1107 1 restriction enzymes. This fragment was used as a PCR template to amplify the SV5 leader sequence. A forward primer was designed which included the first 64 bases of the SV5 leader sequence and a reverse primer was designed to include the first 34 bases of the CAT ORF. The resultant PCR product was digested with Sac1 and Bst 1107 1, gel purified and ligated to the Sac1/Bst 1107 1 fragment from pUCSV5TKCAT which contained the CAT ORF and SV5 trailer sequences. The resultant construct, pUCSV5CAT, was used in subsequent CAT rescue experiments. The negative sense RNA transcript from pUCSV5CAT containing the CAT gene flanked by SV5 leader (le), and trailer (tr), sequences, is referred to as a 'le-CAT-tr' transcript.

1.2.3 Input DNA versus RNA from pUCSV5CAT

The strategy described previously (1.1.1) was used to rescue pUCSV5CAT mRNA from pUCSV5CAT DNA and the resultant CAT assay is shown in Fig.18. Panel A contained extracts of BHK cells which had been infected with VacT7 and SV5, and transfected with increasing amounts of pUCSV5CAT DNA (Lanes 2, 3 and 4 respectively) illustrating detectable CAT enzyme activity. Lane 5 contained a control where 2 µg of pUCSV5CAT DNA was transfected into BHK cells infected with SV5 alone and, as expected, shows no CAT activity. Without VacT7, there would be no -VE sense CAT RNA generated to encapsidate, and from which CAT mRNA could be transcribed. In Lane 6, however, the negative control had detectable CAT enzyme activity from cells transfected with 2 µg of pUCSV5CAT DNA but infected with VacT7 alone. Therefore in the absence of SV5 proteins, CAT enzyme was still generated demonstrating again that VacT7 had the ability to transcribe mRNA from the cDNA construct. This also confirmed that VacT7 was responsible for the CAT activity seen in Lanes 2, 3 and 4.

In an attempt to circumvent the use of VacT7, pUCSV5CAT RNA was transfected in a parallel experiment. The pUCSV5CAT plasmid was linearised with Ear1 and used as the template for an *in vitro* T7 transcription reaction (See Materials and Methods, Chapter 2, Section 3.11 for details). The resultant negative sense RNA transcript was

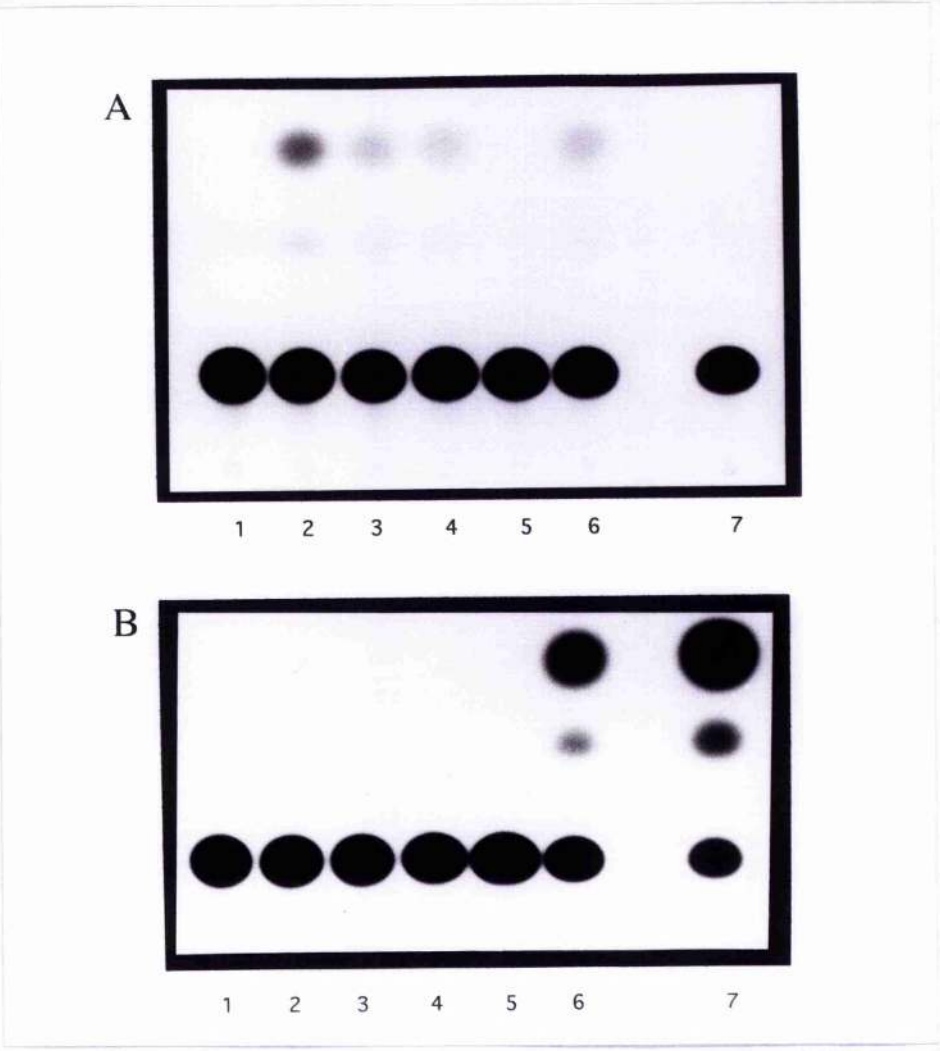


Fig.18:Transfection of pUCSV5CAT DNA or RNA into BHK TK(-) Cells

In Panel A, 500 ng, 1 μg or 2 μg of pUCSV5CAT DNA was transfected into BHK TK(-) cells after infection with SV5 and VacT7 (Panel A; Lanes 2, 3 and 4 respectively). Lanes 5 and 6 contained extracts from cells which had been transfected with 2 μg of pUCSV5CAT DNA after infection with SV5 alone (Lane 5) or VacT7 alone (Lane 6). Lane 1 represents a 'No DNA' control and Lane 7 represents a ^{14}C background control.

In Panel B, 500 ng, 1 μg or 2 μg of pUCSV5CAT RNA was transfected into BHK TK(-) cells after infection with SV5 alone. (Panel B; Lanes 2, 3 and 4 respectively). 2 μg of pUCSV5CAT RNA was transfected into naive BHK TK(-) cells (Panel B; Lane 5) and Lane 1 represents a 'No RNA' control. Lane 6 contained an extract from cells which had been transfected with 100 ng of pSV2CAT control DNA and Lane 8 contained 5 units of CAT enzyme control.

transfected into BHK cells which had been infected with SV5 (Panel B; Lanes 2, 3 and 4), but no CAT activity could be detected (Panel B; Lanes 1 to 5). Lane 6 contained a transfection control demonstrating that the cells had been successfully transfected. Although successful with other similar systems (Luytjes *et al* (1989),.Collins *et al* (1991), Park *et al*, (1991), Pattniak *et al*, (1992)), the rescue of CAT from pUCSV5TKCAT and pUCSV5CAT was unsuccessful using SV5 alone to supply the proteins necessary for encapsidation and transcription of the synthetic transcript. The reason for this is, undoubtedly, multifactorial but one explanation could be due to the synthetic transcript being in direct, and very unfavourable competition with nascent virus genomes and anti-genomes for encapsidation and replication. To overcome this potential problem, the transfection rationale was changed so that SV5 proteins would be generated from cDNA clones and supplied *in trans*, which would remove the necessity for SV5 helper virus.

1.3 Cloning of SV5 NP, P, V and L

An overview of the amended transfection rationale is given in Fig.19, where the proteins thought necessary for encapsidation and subsequent transcription of a synthetic negative sense CAT RNA were to be supplied *in trans* from cDNA clones of the respective genes. The results presented in this section document the construction of an NP clone in the pGEM3Zf(+) vector (Promega), which contained a T7 promoter upstream of the multiple cloning site, facilitating NP protein expression from the VacT7 system.

1.3.1 Construction and expression of pGEM-BLNP

pBR322NP (Paterson *et al*, 1984), was generously provided by Prof. R.A. Lamb (Northwestern University, Evanston, Illinois, USA), where the NP gene had been cloned into the β-lactamase gene of the vector pBR322. This plasmid was used as the template for amplification of the NP ORF by PCR as outlined in Fig.20. (The forward

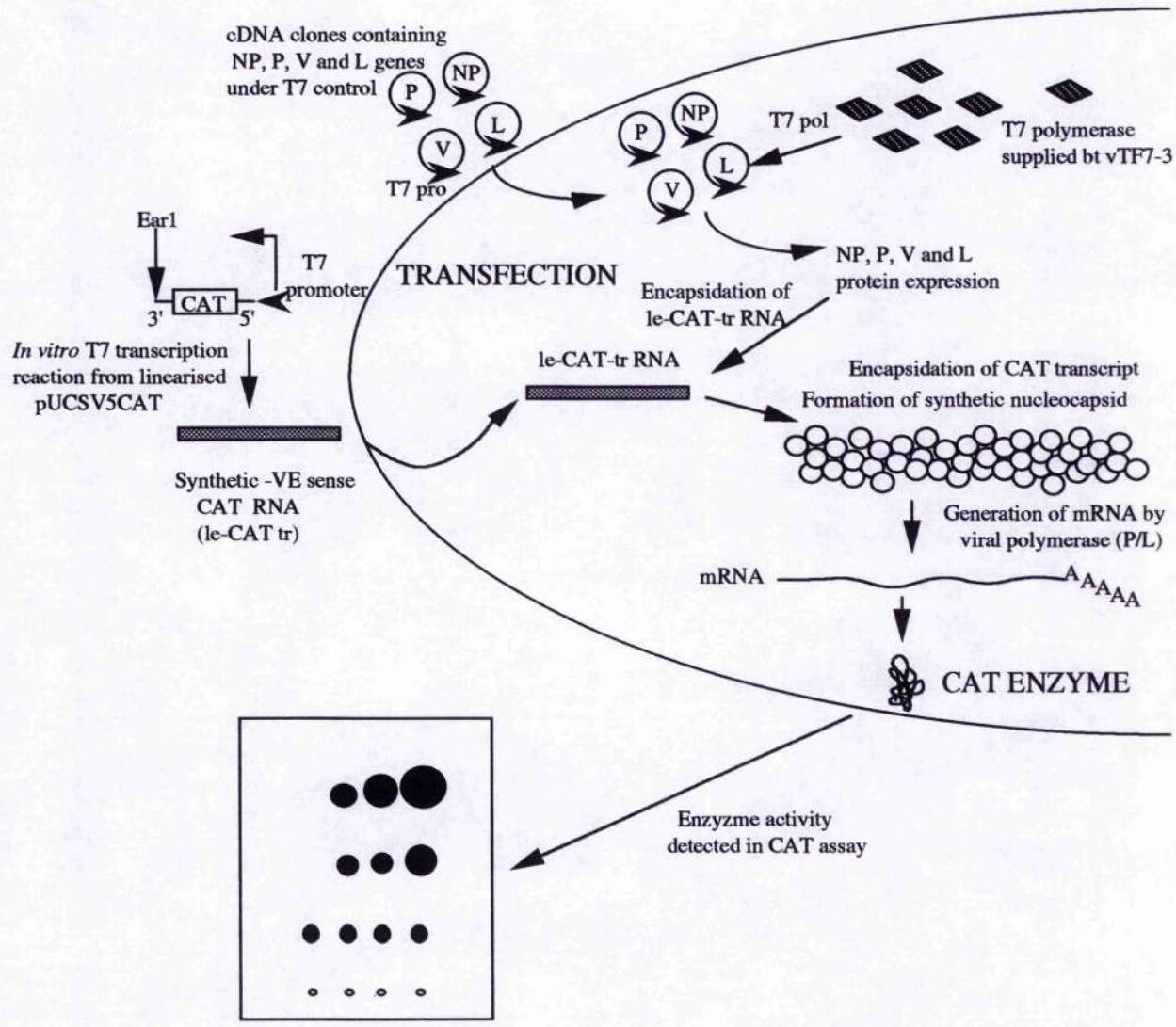


Fig.19:Amended transfection strategy for CAT rescue

Overview of amended transfection strategy for rescue of synthetic CAT transcripts. Negative sense CAT RNA was generated in an *in vitro* T7 transcription reaction from linearised pUCSV5CAT and transfected into cells. Proteins necessary for encapsidation and transcription of the synthetic CAT transcript were supplied by transfecting plasmids encoding SV5 NP, P, V and L genes under the control of the T7 promoter. After encapsidation (formation of a synthetic nucleocapsid) CAT mRNA was to be generated by viral polymerase (P/L) supplied *in trans*, and subsequently translated into CAT enzyme for detection by CAT assay.

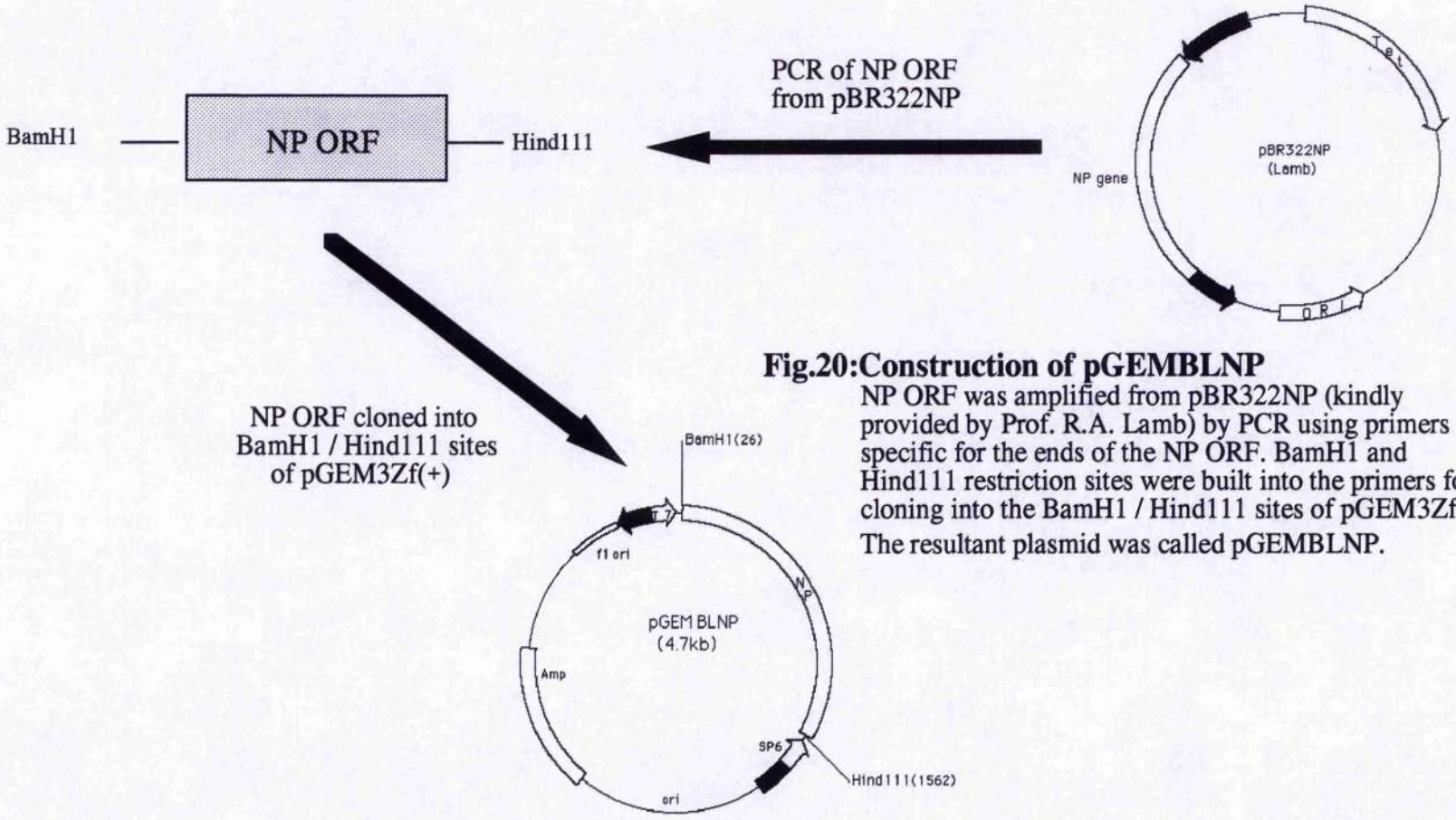


Fig.20:Construction of pGEMBLNP

NP ORF was amplified from pBR322NP (kindly provided by Prof. R.A. Lamb) by PCR using primers specific for the ends of the NP ORF. BamH1 and Hind111 restriction sites were built into the primers for cloning into the BamH1 / Hind111 sites of pGEM3Zf(+). The resultant plasmid was called pGEMBLNP.

PCR primer contained the first 21 bases of the NP ORF and encoded the recognition sequence for the restriction enzyme BamH1. The reverse primer contained the last 24 bases of the NP ORF and the recognition sequence for the restriction enzyme Hind111.) Once the NP gene was amplified, the PCR product was digested with BamH1 and Hind111 restriction enzymes and run on a 1% agarose gel for band purification. (See Materials and Methods, Chapter 2, Section 3.5) The purified NP ORF was ligated to the BamH1 / Hind111 sites of pGEM3Zf(+) which had been digested and purified in parallel to the NP ORF. The resultant construct was called pGEM-BLNP.

It should be added here that there were very few transformants from this ligation step which was repeated many times before 4 clones were generated. These transformants grew very slowly on agar plates and in liquid culture, and gave poor plasmid yield if grown for less than 48 hours.

Expression of NP protein from pGEM-BLNP was tested by transfecting 4 clones into 293 cells infected with VacT7. (Note that 293 cells were used here, as the transfection efficiency of this cell line had been shown to be much higher than BHK cells.) At 18 hours post-infection cells were harvested for western blot analysis shown in Fig.21. Lanes 1, 2, 3 and 4 contained the pGEM-BLNP clones and all expressed a truncated NP protein of around the same size as SV5 P protein (Compare Lanes 1-4 with ICE). Lanes 5 and 6 contained negative controls of extracts from 293 cells infected with VacT7 (Lane 5), or naive 293 cells (Lane 6), demonstrating that the major band in each lane which ran with approximately the same mobility as NP was due to a non-specific cross-reaction of the mAbs with host cell protein. However, there did seem to be some low level expression of full-length NP from the clone in Lane 3. This clone also produced a protein of around the same mobility as P protein, (43 kDa), suggesting that there may have been some internal initiation of translation. It was calculated that if the ninth AUG codon was used as the initiation codon, then a protein of 42 kDa would be generated. The small amount of full-length NP being generated could be explained by there being a low frequency of correct initiation at the first AUG. A second explanation for the generation of a truncated NP protein from pGEM-BLNP was much simpler. If the clone contained a mutation which led to a premature termination codon, then a truncated

protein would be inevitable. To distinguish between these possibilities, the entire NP ORF was sequenced.

1.3.2 Sequencing of pGEM-BLNP

The NP ORF was sequenced and apart from 2 silent mutations at positions 732 and 1074 of NP ORF (both C-T transitions), the NP sequence was found to be identical to that published by Parks *et al*, (1992), until base 1232 of the ORF. Fig.22 shows that after base 1232 of the NP ORF, there were a number of C residues followed by an anti-sense sequence from the β-lactamase gene. The original template used to PCR amplify the NP ORF was pBR322NP (Paterson *et al*, 1984), in which the NP gene had been cloned into the β-lactamase gene of pBR322. The reason for amplification of β-lactamase sequence as part of the NP ORF remains unclear. When initially amplified, the resultant PCR product was the expected molecular weight of the entire NP ORF and no complementary region to the NP reverse primer was found whilst sequencing this clone. Due to the difficulty in initially generating this clone and that 1232 nucleotides (from a total of 1532) had been sequenced demonstrating no coding changes to the published sequence, it was clear that a C-terminal repair of the existing clone was more appropriate than the generation of an entirely new NP clone.

1.3.3 Carboxy-terminal repair of pGEM-BLNP

A schematic diagram of the cloning strategy employed to repair the carboxy-terminus of NP is shown in Fig.23. The NP ORF was amplified by RT/PCR from NP mRNA in total RNA prepared from SV5 infected Vero cells using the same oligonucleotide primers used in the amplification from pBR322NP. The resultant PCR product was digested with Mun1 and Hind111 restriction enzymes. The C-terminal Mun1-Hind111 fragment (450 base pairs) was purified from the rest of the NP ORF by running on a 1%

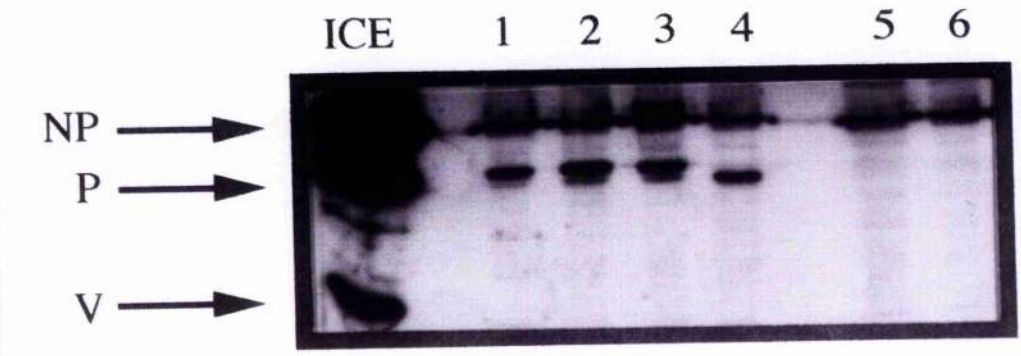


Fig.21:Expression of NP protein from pGEM vectors

293 cells were infected with VacT7 and transfected with NP clones and protein expression was analysed by western blot. 293 cells were plated in 6 well dishes and infected with VacT7 at m.o.i. of 1. At 1 hour post-infection, cells were transfected with 1 µg of DNA from 4 pGEM-BLNP clones and harvested 18 hours post infection for analysis by western blot. Lanes 1, 2, 3 and 4 show the expression from the pGEM-BLNP clones. Lane 5 and 6 were negative controls containing cell extracts from untransfected VacT7 infected 293 cells and uninfected 293 cells respectively. An SV5 infected cell extract (ICE) indicated the migration of the polypeptides in the polyacrylamide gel. NP protein was detected on the western blot with the polyclonal anti-NP antibody while the P and V proteins were detected with the mAb SV5-P-k. Bound antibodies were detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig.

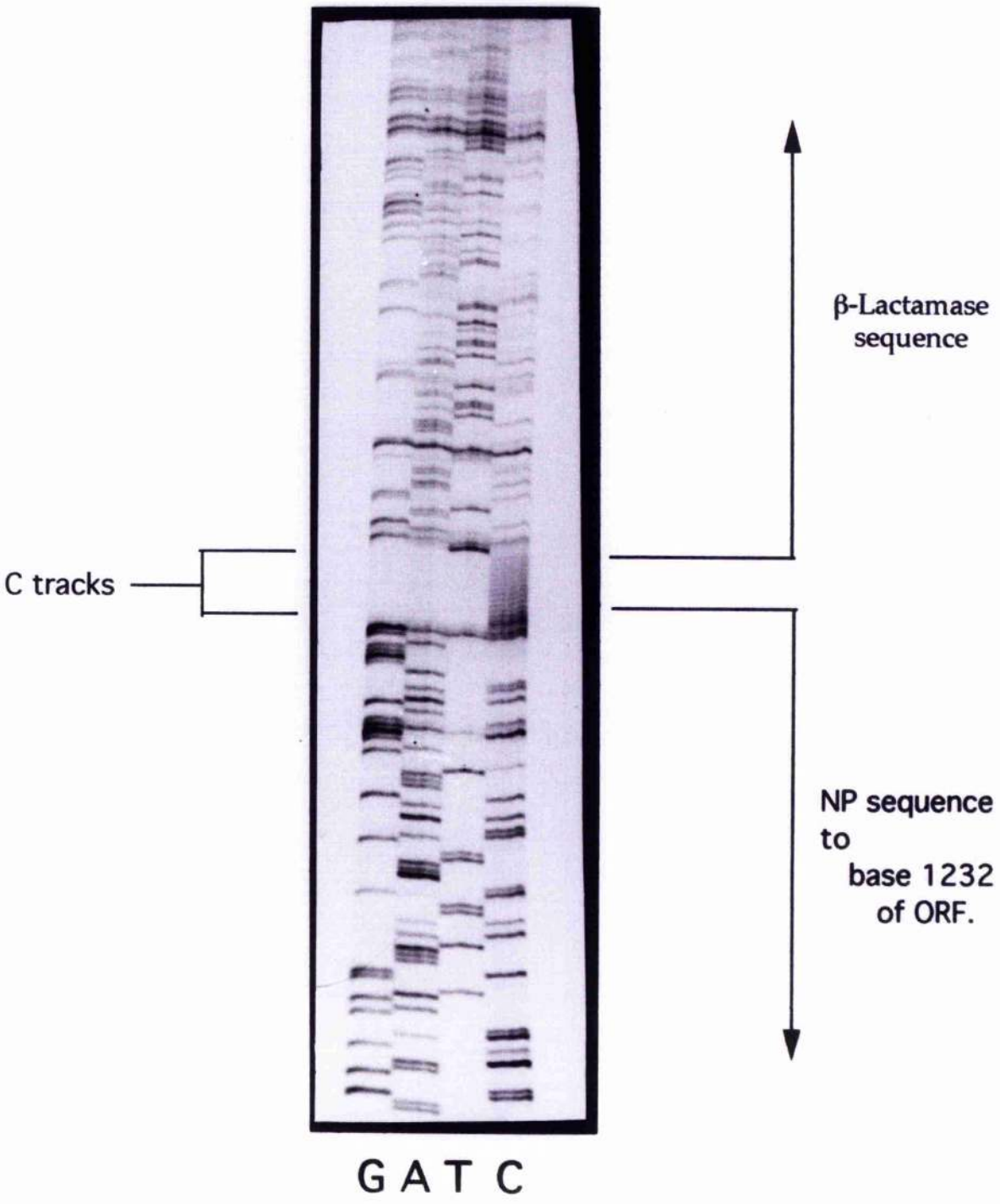


Fig.22: Sequencing of pGEMBLNP

Sequence of pGEMBLNP is shown to base 1232 of NP ORF. A stretch of C residues indicate the end of NP sequence and the beginning of the β-Lactamase gene sequence corresponding to base 1617 on the anti-parallel strand of pGEM3Zf(+).

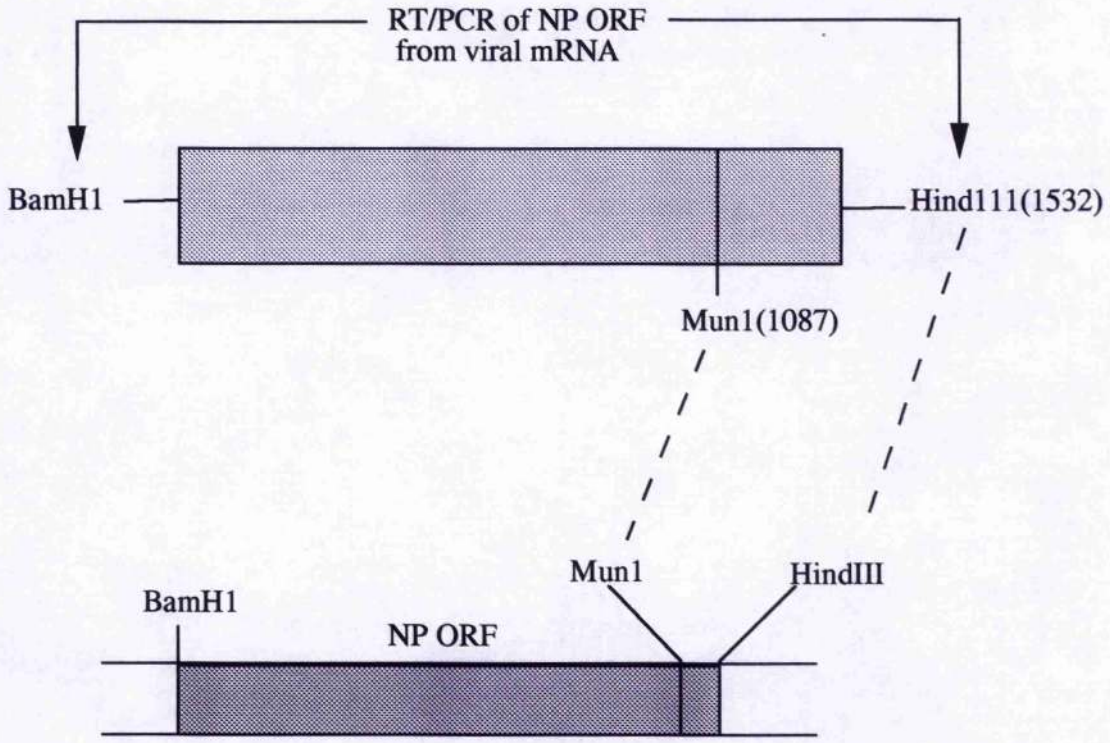


Fig.23 : C-terminal repair of pGEMBLNP

The C-terminus of the NP clone was found to be incomplete and needed to be replaced. The whole of the NP ORF was amplified from viral mRNA from SV5 infected Vero cells in an RT/PCR reaction. Both pGEMBLNP and the resultant cDNA encoding the NP ORF were digested with Mun1 and HindIII and ligated together to form pGEMBLNP(Repaired).

agarose gel and excising the band of interest for gel purification. pGEM-BLNP was also digested with Mun1 and Hind111 and purified away from the small C-terminal fragment as above. The C-terminus generated by PCR, was ligated to pGEM-BLNP and and the resultant plasmid was called pGEM-BLNP(Repaired). The clone was sequenced and it was confirmed that no point mutations in the new C-terminus had been introduced by PCR. Fig.24 shows the sequence from pGEM-BLNP(repaired) using an oligonucleotide primer which bound at bases 1015-1029 of NP ORF. The sequence shown corresponds to bases 1119 -1260 of the NP ORF and covers the region where the stretch of C residues (position 1232) led into the β-lactamase gene before the repair. Protein expression from the repaired clone was then examined.

1.3.4 Increase in NP expression after the addition of the β-Globin leader sequence

During the initial expression of the NP clones in *in vitro* transcription / translation reactions, it was noted that protein expression levels were low (Data not shown). Analysis of *in vitro* synthesised proteins is frequently hampered by inefficient translation, especially of large proteins. It had been demonstrated that replacement of the GC-rich leader of human serum response factor (SRF) by an in frame fusion to the human β-globin leader sequence including the AUG initiation codon, strongly stimulated translation in a reticulocyte lysate (Norman *et al*, 1988). This observation was expanded to show that the region homologous to the 5' end of the rabbit β-globin RNA, but lacking the initiation AUG, could enhance translation efficiencies in both rabbit reticulocyte lysates and in wheatgerm extracts (Annweiler *et al*, 1991).
In an attempt to increase the amount of NP expressed from pGEM-BLNP, the rabbit β-globin leader sequence was added as shown in Fig.25. Complementary oligonucleotide primers were designed which encoded the entire rabbit β-globin leader sequence, the first 30 bases of the NP ORF and restriction enzyme recognition sequences for EcoR1 (forward primer) and Nde1 (reverse primer). These primers were used to amplify the β-globin leader region by PCR in a 'primer dimer' reaction due to complementarity of the

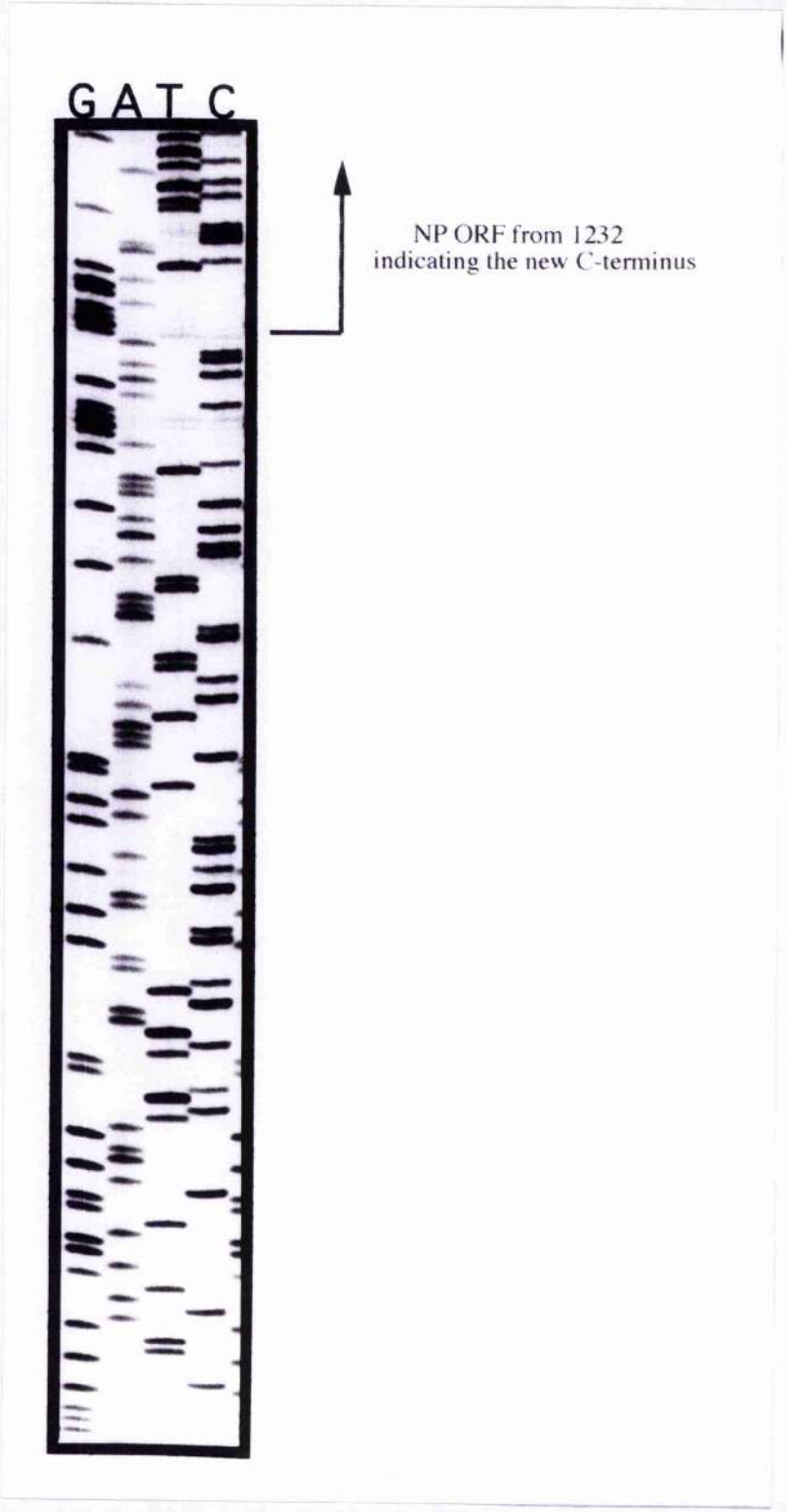


Fig.24: Sequencing of pGEMBLNP-end repaired

Sequence from bases 1120-1260 of NP ORF from C-terminally repaired pGEMBLNP. Arrow indicates bases 1232 onwards where β-lactamase sequence was replaced by new NP C-terminal region.

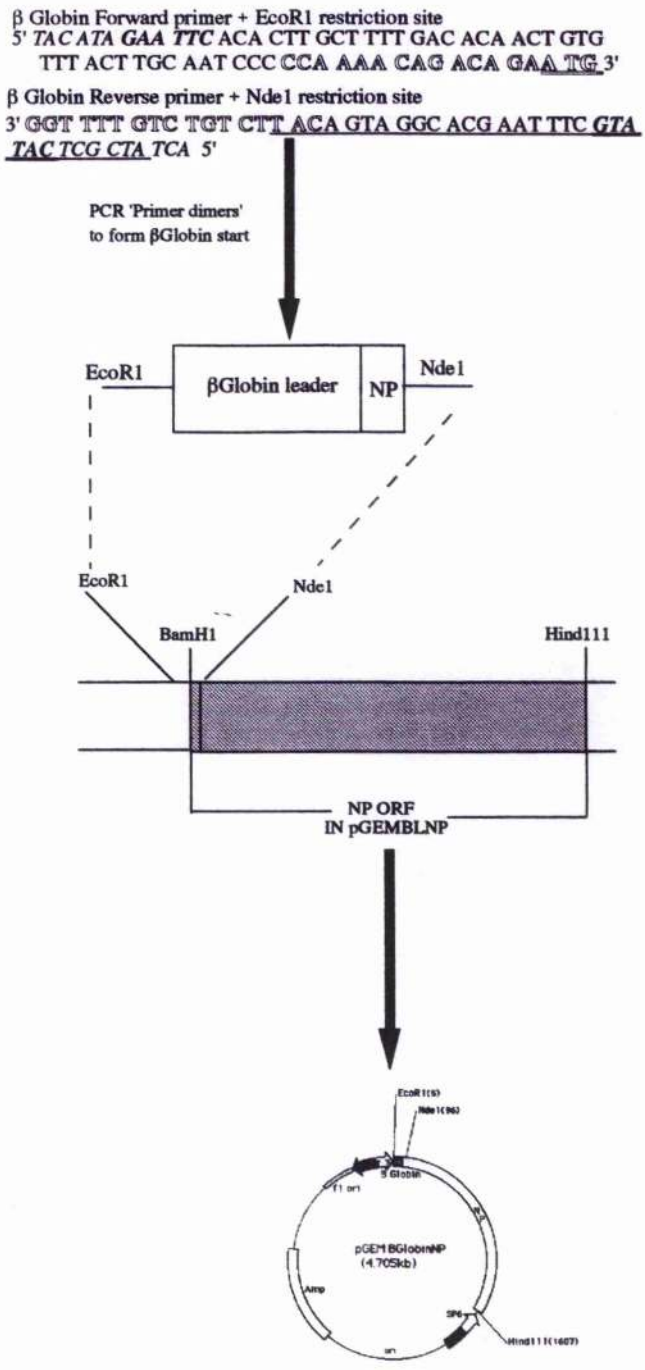


Fig.25:Construction of pGEMβGlobinNP

To ensure translational initiation at the first AUG of the NP ORF, the rabbit βGlobin untranslated leader region was added to the start of the NP ORF of pGEMBLNP. The βGlobin leader region was amplified by using complementatry primers encoding the entire region including the first 30 bases of the NP ORF in PCR 'primer dimer' reaction. Complementary regions of the primers are outlined, restriction enzyme sites are in bold and sequence in the NP ORF is underlined. The resultant PCR product and pGEMBLNP were digested with EcoR1 and Nde1 and ligated together to form pGEMβGlobinNP.

primers. The resultant PCR product, along with pGEM-BLNP, were digested with EcoR1 and Nde1. (Note that the cloning procedure took advantage of a unique Nde1 site at base number 30 of the NP ORF allowing fusion of the β-globin leader region to the initiating ATG of the NP ORF.) Once digested, DNA fragments were separated by running on a 1% agarose gel whereupon the bands of interest were excised, purified and ligated. The resultant plasmid was called pGEM-βGlobinNP and NP expression from this clone was analysed by *in vitro* transcription/translation (See Materials and Methods, Chapter 2, Section 5.3 for details).

pGEM-βGlobinNP was linearised by digestion with Hind111 restriction enzyme and 1 μg of linearised plasmid was used as the template for *in vitro* transcription/translation. An mRNA transcript was first generated by T7 DNA-dependent RNA polymerase and subsequently translated by rabbit reticulocyte lysate which utilised ^{35}S- methionine to generate radiolabelled translation products which were separated by SDS-PAGE. The gel was dried and put against X-ray film for autoradiography. Fig.26 Lane 3, demonstrated that the migration of the truncated NP protein before C-terminal repair, was slightly higher than that of the P protein (Lane 4), but after C-terminal repair pGEM-BLNP(Repaired) expressed full length NP (Lane 2). The effect on translation of the β-globin enhancer region was seen in Lane 1 which contained pGEM-βGlobinNP and demonstrated an increased level of translated product compared to pGEM-BLNP (Lane 2). Therefore, pGEM-βGlobinNP was used in all future experiments and was used as the parental plasmid for all future cloning steps.

1.3.5 Cloning of other SV5 proteins

As part of the reverse genetics project, the cloning of the SV5 genes was carried out in collaboration with Mr. B.L. Precious who constructed and generously provided pGEM-P and pGEM-V where the P and V ORFs had been cloned into pGEM3Zf(+). Once constructed, pGEM-P and pGEM-V ORFs were completely sequenced by me. Two changes to the published sequence of Thomas *et al*, (1988) were found in the P ORF (A-C substitution at position 207 of ORF, and A-G substitution at position 993 of

Fig.26:Expression of NP protein after C-terminal repair and addition of βGlobin leader sequence

NP protein expression from pGEM vectors was analysed by *in vitro* transcription/translation after C-terminal repair and addition of the βGlobin leader sequence. 1 μg of linearized plasmid DNA was used as the template for ^{35}S methionine labelled *in vitro* transcription / translation reactions. From a 25 μl reaction, 5 μl was added to 15 μl lysis buffer, boiled and 10 μl (equivalent of 10% of translation mix) loaded on a 10 % gel for electrophoresis. Gel was dried and put against film for autoradiography. Lanes 1 and 2 show full length NP expressed from pGEMβGlobinNP and pGEMBLNP after C-terminal repair. Lane 3 shows NP expression from pGEMBLNP before the C-terminal repair. A comparison of Lanes 1 and 2 demonstrates the effect of the βGlobin enhancer region on NP expression levels from pGEMβGlobinNP and pGEMBLNP respectively.

ORF), neither of which led to an amino acid change. One change from the published sequence was found in the V ORF (A-G substitution at position 466 of ORF) which led to a change in amino acid number 156 from a Serine to a Glycine residue (AGT (Ser)-GGT (Gly)).

Although many attempts at constructing a pGEM-L clone were made, a full length clone proved elusive and therefore the clone pGEM3-L was kindly provided by Prof. R.A. Lamb (Northwestern University, Evanston, Illinois, USA). The construction of the L clone into pGEM3 (Promega) is documented in Parks *et al*, (1992), where 2 independent clones were completely sequenced without finding an amino-acid difference. However, during the course of the rescue experiments, there remained a doubt over the ability of the polymerase clone to generate mRNA since at the time these experiments were carried out, the L clone had not been shown to be functional.

1.4 Addition of SV5 proteins *in trans*

The pGEM constructs containing the SV5 genes to be used for CAT rescue were found to be incompatible for co-transfection experiments. This was due to NP, P and V having been cloned into pGEM3Zf(+) vector, whereas L (having been supplied by Prof. R.A. Lamb), had been cloned into the pGEM3 vector. The fundamental difference between vectors was the orientation of the T7 promoter with respect to the multiple cloning sites. The position of the T7 promoter in pGEM3Zf(+) gave rise to a "clockwise" mRNA species whereas pGEM3 gave rise to an "anti-clockwise" mRNA species. Since this was the only difference between the vectors, mRNAs generated from co-transfected vectors had complementary mRNA tails which could inhibit translation. This was overcome by subcloning the individual genes into another vector which gave rise to mRNA transcription in one direction. The vector system chosen also increased protein production from the VacT7 system.

1.4.1 Cloning of SV5 genes in pTM-1

Recombinant vaccinia virus (VacT7), as with all poxviruses, encodes its own capping, methylating and polyadenylating enzymes, so it was anticipated that that the transcripts made by the T7 polymerase would be properly modified for stability and translation. Fuerst and Moss (1989) found that although the amounts of RNA generated by VacT7 were very high, (30% of total RNA in the cytoplasm), analysis of the RNA indicated that only 5% was capped and methylated. Since the cap structure is required for efficient translation in eukaryotic cells, only moderate levels of protein synthesis were achieved. Moss *et al* (1990) utilised the untranslated region of the picornavirus, encephalomyocarditis virus (EMCV), to generate T7 transcripts which were cap-independent for translation. Unlike cellular mRNA or mRNA from other viruses, picornavirus RNAs do not contain cap-structures but instead, have a long untranslated region (UTR) that facilitates ribosome binding (Jang *et al*, 1988). Elroy-Stein *et al* (1989) placed the EMCV UTR just downstream of the T7 promoter and found protein expression levels increased 5-10 fold.

The plasmid pTM-1 was constructed by Moss *et al* (1990) to express genes under the control of the T7 promoter / EMCV UTR. The gene of interest was inserted into the multiple cloning site such that the Nco1 site provided the translation initiation codon. This plasmid was utilised to increase the level of NP, P and V being expressed in the VacT7 system for CAT rescue. The SV5 P and V genes were sub-cloned from pGEM-P and pGEM-V as outlined in Fig.27. Both pGEM-P and pGEM-V had been generated by RT/PCR using the same oligonucleotide primers. The design of the forward primer fortuitously created a Nco1 site at the initiating ATG of each ORF allowing direct sub-cloning into pTM-1. Both parental plasmids (pGEM-P and pGEM-V) were digested with Nco1 and Sal1 restriction enzymes. The P and V ORFs were separated from the parental vector by running on a 1% agarose gel and excising the bands of interest for purification. The recipient vector, pTM1, was digested with Nco1 and Xho1 restriction enzymes, purified as before, and ligated to either P or V ORFs. (Note that Sal1 and Xho1 digested DNA have compatible ends which ligate to form a Taq1 restriction site.) The resultant plasmids were called pTM-P and pTM-V respectively. Similarly, a pTM

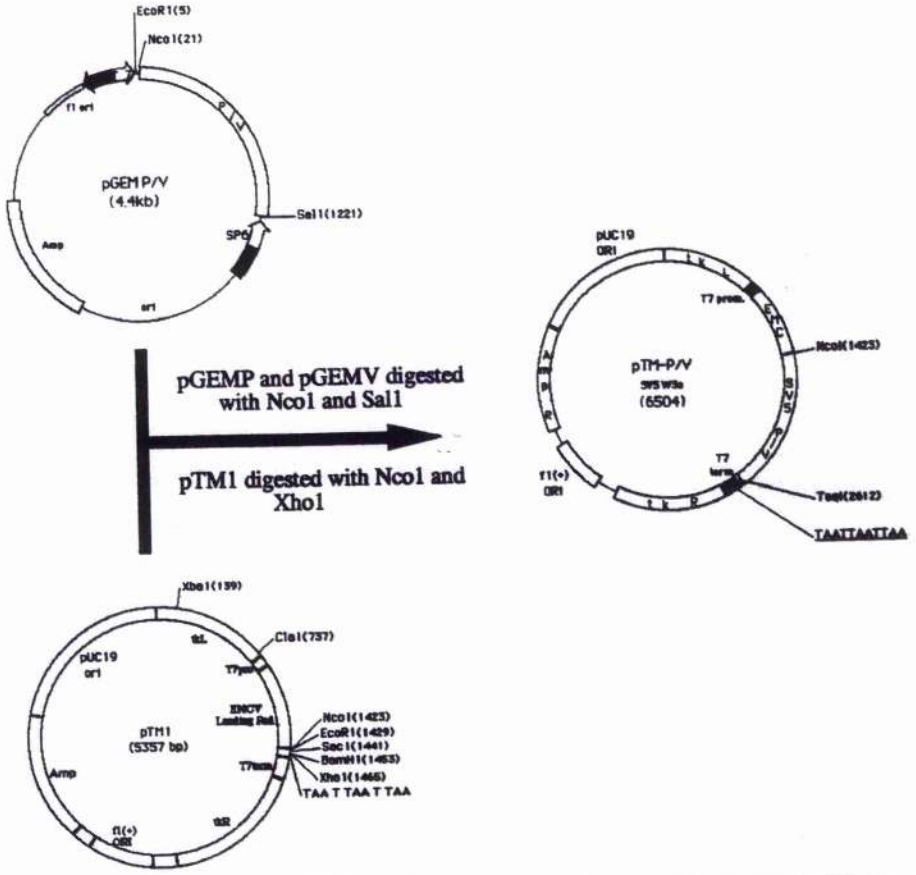


Fig.27:Construction of pTM-P and pTM-V

To increase the translation properties of T7 transcripts, the SV5 P and V ORFs were cloned into pTM1 which adds the EMCV UTR at the initiating AUG. pGEM-P and pGEM-V(represented as pGEM P/V) were digested with Nco1 and Sal1 restriction enzymes to isolate the P and V ORFs and ligate them into pTM1 which had been digested with Nco1 and Xho1. The restriction enzymes Sal1 and Xho1 have compatible ends which ligate together to form a Taq1 site. The resultant plasmids were called pTM-P and pTM-V (represented as pTM-P/V)

clone expressing NP was obtained but a full length pTM-L clone proved elusive and so the pGEM-L clone (compatible with the pTM system) was used.

1.4.2 Co-transfection of NP, P, V and L

Fig.28 shows the expression of NP, P, V and L (from pTM-NP, pTM-P, pTM-V and pGEM-L) in 293 cells infected with VacT7 and immunoprecipitated (See Materials and Methods, Chapter 2, Section 5.4 for details) with mAbs SV5 P-k (P and V proteins), SV5 NPa (NP protein) and anti-L (N-terminal) peptide serum. Labelled polypeptides were separated by elecrophoresis through a 10% SDS-polyacrylamide slab gel which was subsequently dried and put against X-ray film for autoradiography.

Lanes 2, 3, 4 and 5 show NP, P, V and L expression from their respective cDNA constructs. Lane 6 demonstrated NP+P co-expression from co-transfection of pTM-P and pTM-NP. However, when NP+P+V were cotransfected, there was a decrease in the amount of NP and P expressed, and the V protein itself was not detected (Compare Lanes 6 and 7). This observation was repeated in Lane 8 where NP+P+V+L were cotransfected. L was expressed at similar levels when co-expressed as it had been when expressed alone (Lanes 5 and 8), but levels of NP and P were still decreased (Compare lanes 6 with Lanes 7 and 8) and V was again not detected.

This result, although somewhat curious, was reproducible. Therefore, the experiment was repeated and the (unlabelled) immunoprecipitated samples were analysed by western blot to increase sensitivity of detection (Fig.29). NP, P, V and L were expressed alone in Lanes 4, 5, 6 and 7 respectively. (Note that anti-L peptide serum worked extremely poorly in western blot and so was omitted from the detection step. L was therefore not detected in this figure and so Lane 7 merely shows sample spilled from Lane 8.) This experiment showed that V was indeed being made when co-expressed with NP and P but to a lesser degree than when expressed alone. The decrease in NP and P expression on the addition of V was again seen (Compare Lane 8 with Lanes 11 and 12) suggesting that V may have been having a negative effect on the ability of the

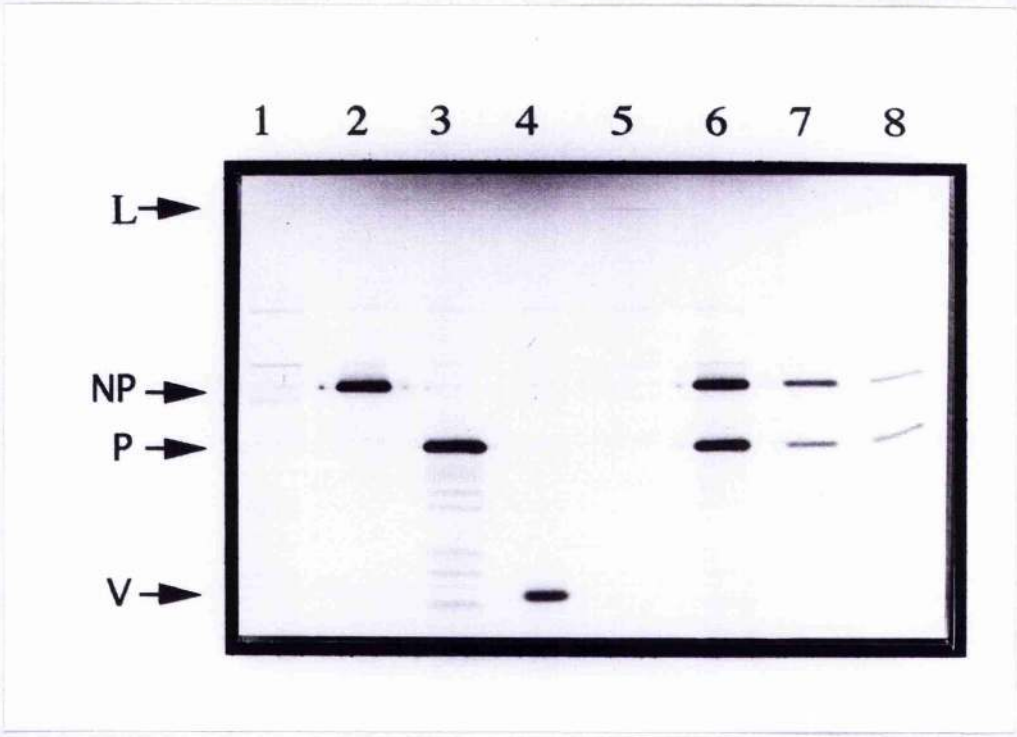


Fig.28:Transient SV5 protein expression in VacT7 system

IP demonstrating co-expression of SV5 proteins from co-transfection of pTM-NP, pTM-P, pTM-V and pGEM-L in 293 cells infected with VacT7. 1 μg of each plasmid DNA was transfected into 293 cells either singly or in combination 1 hour post VacT7 infection. Cells were labelled with ^{35}S methionine (17 hours post VacT7 infection) for 1 hour prior to immunoprecipitation. Extracts were precipitated with mAbs SV5-NPa, SV5-Pk (for both P and V proteins) and anti-L (N-terminal) peptide antiserum. Samples were loaded onto 10% polyacrylamide gel for electrophoresis.and subsequently dried and put against film for autoradiography. Lanes 2, 3, 4 and 5 represent NP, P, V and L expressed alone. Lane 6, 7 and 8 represent NP+P+V, NP+P and NP+P+V+L respectively. Lane 1 was a negative control of VacT7 infected cell extract which was incubated with a pool of the antibodies mentioned above.

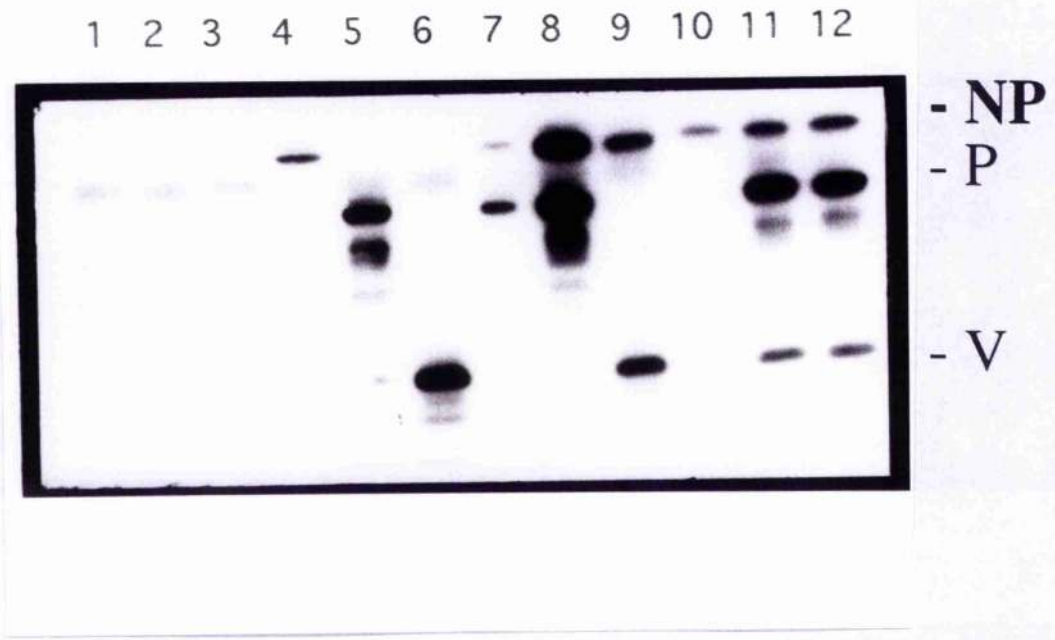


Fig.29: Western Blot of SV5 protein co-expression

Western blot of immunoprecipitated SV5 proteins in co-expression experiment from co-transfected 293 cells. Repeat of transfection shown in Fig.28, where samples were immunoprecipitated and western blotted. Lanes 4, 5, 6 and 7 represent NP, P, V and L expression from pTM-NP, pTM-P, pTM-V and pGEM-L respectively. NP+P were co-expressed in Lane 8, NP+V in Lane 9, NP+L in Lane 10, NP+P+V in Lane 11 and NP+P+V+L in Lane 12. Negative controls were run in Lanes 1, 2 and 3 from uninfected 293cells, VacT7 infected 293 cells and VacT7 infected 293 cells and transfected with pTM1 vector respectively. The polypeptides were detected with mAbs SV5-NP-d, and SV5-P-k (which detects both P and V proteins). Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig.

proteins to be immunoprecipitated. These findings were not examined more closely at this stage. Co-expression of NP, P, V and L had been demonstrated in the VacT7 system but functionality of the recombinant proteins had not been examined. This was tested by their ability to encapsidate and transcribe pUCSV5CAT negative sense RNA into mRNA.

1.4.3 Construction of pPanHan for rescue

SV5 proteins necessary for encapsidation and transcription of le-CAT-tr RNA were supplied *in trans* from co-transfection of pTM-NP, pTM-P, pTM-V and pGEM-L into VacT7 infected 293 cells as demonstrated in Section 1.4.2. The le-CAT-tr RNA was generated by an *in vitro* T7 transcription reaction from pUCSV5CAT linearised by digestion with EarI (as in Section 1.2.3). No CAT activity was detected in attempts to rescue CAT mRNA from input le-CAT-tr RNA (Data not shown).

The focus of the rescue project was then transferred to the laboratory of Prof. R. A. Lamb, Northwestern University, Evanston, Illinois, USA, where, in collaboration with Prof. Lamb and Dr. Carol Ward, a fresh approach was taken. Dr. Ward had performed experiments similar to those described above, independently to me, and also without success. In discussion with both Dr. Ward and Prof. Lamb, it was agreed that a 'pan-handle' construct should be constructed to aid transciption initiation of the genome analogue.

It had been reported that initiation of influenza virus transcription from genomic RNA relied on the formation of panhandle structures from the the 3' and 5' terminal nucleotides of virion RNA segments (Fodor *et al*, (1994), Pritlove *et al*, (1995)). The "panhandle" or "fork" regions were shown to contain highly conserved non-coding nucleotides which were important for the transcriptional activity of the viral polymerase. This strategy was employed for CAT rescue by generating a cDNA construct which would give rise to a CAT RNA species with complementary 3' and 5' termini which formed pan-handle structures. Fig.30 shows a schematic diagram of the construction of cDNA clone encoding such a panhandle, pPanHan. This was

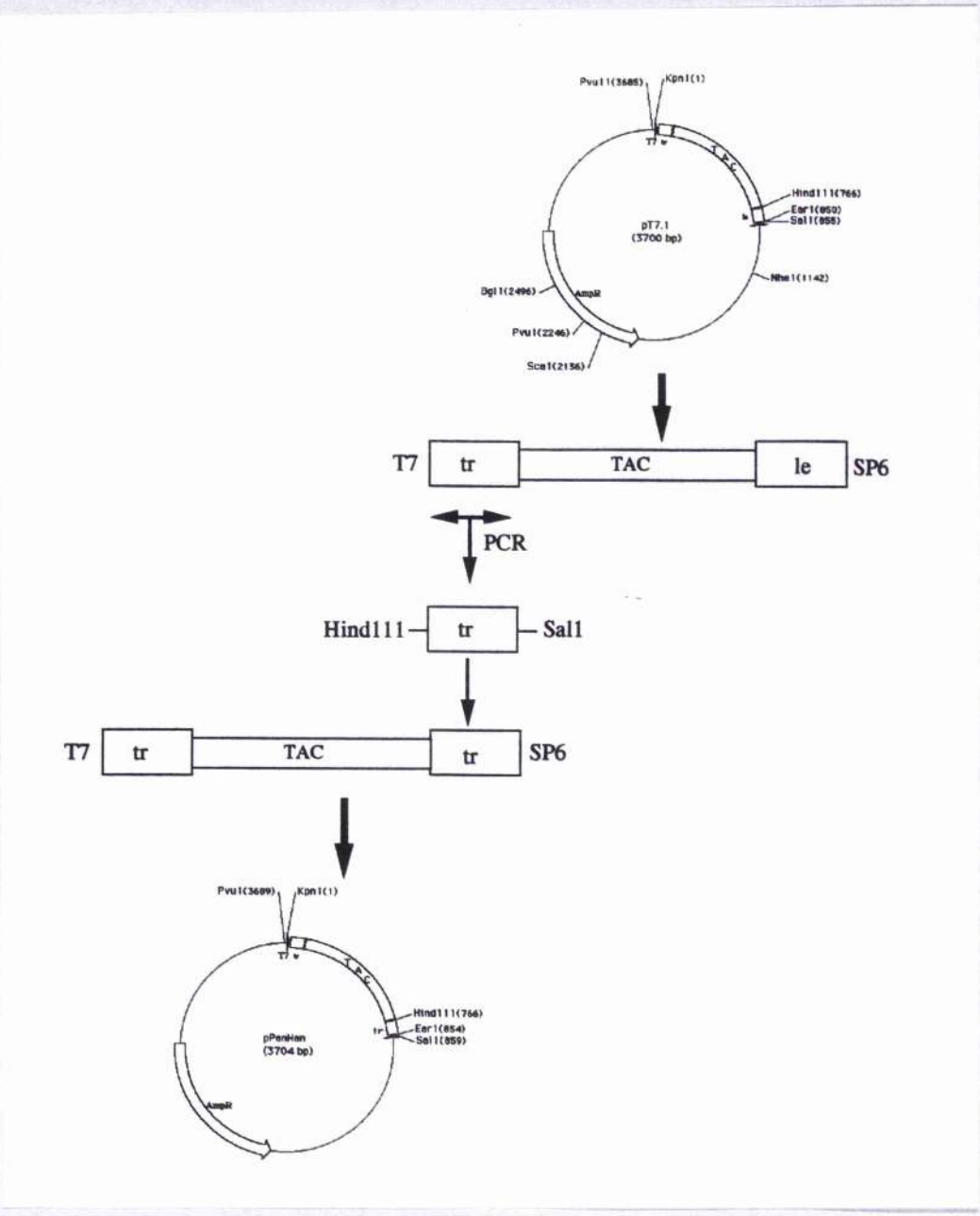


Fig.30:Construction of pPanHan

To increase potential for replication of the synthetic CAT transcript, modifications were made to the cDNA clone resulting in a pan-handle structure for the synthetic transcript. pT7.1 tr-TAC-le contsruct (provided by Dr. C. Ward, Northwestern University, Illinois, USA) was used as a PCR template to amplify the SV5 trailer region. The primers used encoded Hind111 and Sal1 restriction enzyme sites which allowed the PCR product to replace the leader region from pT7.1. Both the PCR product and pT7.1 were digested with Hind111 and Sal1, ligated together and the resultant plasmid was called pPanHan.

constructed from pT7.1, a le-CAT-tr construct provided by Dr. Ward which also obeyed the rule of six (Chapter 1, Section 2.2.2). Oligonucleotide primers were designed to amplify the SV5 trailer region by PCR. The primers used contained Hind111 and Sal1 restriction enzyme sites which allowed the PCR product to replace the existing leader sequence in pT7.1. Following digestion of both the PCR product and pT7.1 with Hind111 and Sal1 restriction enzymes, fragments were separated by running on a 1% agarose gel, excised and purified. The fragments were then ligated together and the resultant plasmid was called pPanHan.

In Fig.31, large scale lithium chloride DNA preparations (See Materials and Methods Chapter 2, Section 3.1 for details), of four pPanHan clones were digested with Hind111 and Sal1 restriction enzymes to confirm the leader sequence (le) had been replaced by the trailer sequence (tr). Due to the small sizes of the le (89 base pairs) and tr (47 base pairs) regions, digests, including one of the parental pT7.1 digested in parallel, were run on an 8% polyacrylamide slab gel and stained with ethidium bromide. Lane 6 contained the parental pT7.1 plasmid digest where the 89 base pair le sequence was visible. Lanes 2, 3 and 5 clearly showed a 47 base pair fragment had been excised from clones 1, 2 and 4 indicating the tr region had indeed replaced the le region. Lane 4 showed that the remaining clone was a religated plasmid which did not contain either the original le or the new tr region. Clone 1 (Lane2) seemed also to have a slight band corresponding the original parental le as well as the newly added tr and so was discarded. Clone 4 (Lane 5) was chosen as pPanHan and sequenced, confirming no PCR errors had been introduced.

pPanHan was linearised by digestion with Sal1 and used as a template for the generation of negative sense tr-CAT-tr RNA by *in vitro* transcription. The tr-CAT-tr RNA was transfected into 293 cells along with plasmids encoding the SV5 proteins necessary for encapsidation and transcription of the synthetic RNA and assayed for CAT activity as before. No CAT activity was detected using pPanHan, pT7.1 or pUCSV5CAT RNA as the input RNA for these assays.

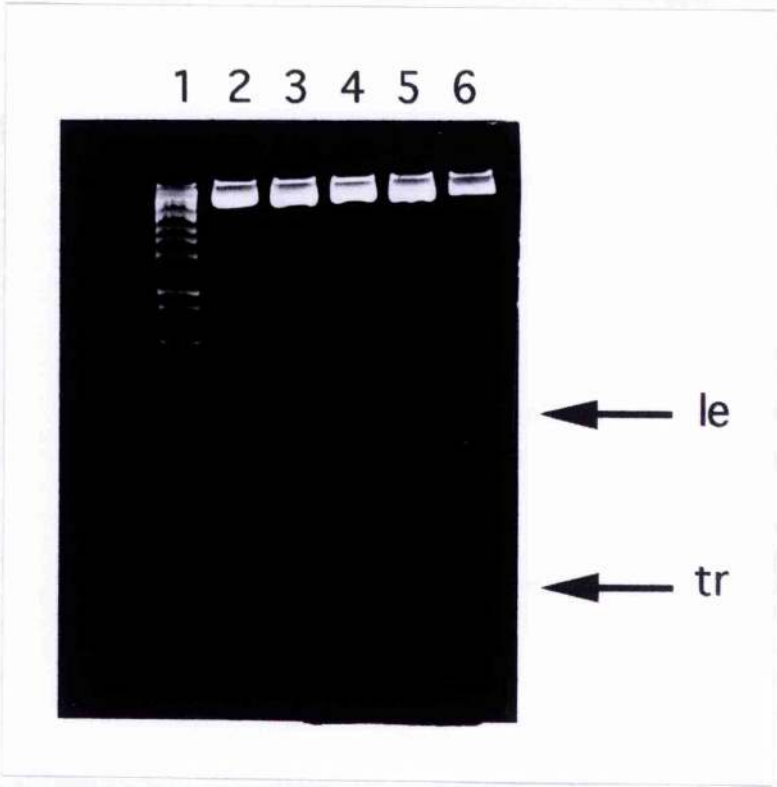


Fig.31:Digestion of pPANHAN constructs

Diagnostic restricion digests to confirm construction of pPANHAN before sequencing. DNA from clones 1-4 were prepared by the LiCl method and digested with Hind11 and Sal1 restriction enzymes (Lanes 2-5 respectively) Lane 6 contained parental pT7.1 DNA digested with the same enzymes. Lane 1 contained Hinf1 digested pM45 as a molecular weight ladder. All samples were run on an 8% acrylamide gel and stained with ethidium brimide. SV5 le (89 bases) and tr (47 bases) are indicated.

Although levels of SV5 proteins expressed by the VacT7 system were manipulated to mimic molar ratios of proteins seen in an SV5 infection, no effect was seen in the results of the CAT rescue experiments which all showed no detectable CAT enzyme activity. Supplying the SV5 proteins by SV5 infection or *in trans* from cDNA clones driven by VacT7, proved unsuccessful in attempts to rescue CAT mRNA transcripts. In parallel to the CAT enzyme assays, cell extracts from the transfected cells were subjected to Northern Blot analysis which also failed to detect CAT mRNA (Data not shown). One possible explanation could have been that the template RNA had not been successfully transfected into the 293 cells. However, in control experiments, *in vitro* transcribed luciferase mRNA was successfully transfected as judged by the detection of luciferase enzyme activity 18 hours post-transfection (Data not shown). This indicated that the transfection method used (cationic liposomes) was also a successful RNA delivery system.

There could be two possible explanations for the failure of the le-CAT-tr rescue system. Firstly, the SV5 proteins may have failed to encapsidate the transfected RNA. If nascent vRNA (genomic and antigenomic) is normally held in a conformation favourable for initiation of encapsidation, maybe such a conformation was not achieved by the synthetic transcript. Secondly, the synthetic transcript may have been encapsidated, but perhaps failed to generate a synthetic nucleocapsid structure which was recognisable to the viral polymerase. At the time of these studies, functional analyses of the cloned viral polymerase had yet to be performed so even if the synthetic transcript had been encapsidated and formed a synthetic nucleocapsid structure, there remained a doubt over the ability of the polymerase clone to generate mRNA.

Since the rescue system had been unsuccessful to this point, an added avenue of investigation was neccessary to allow expansion of the overall focus of the project. Development of inducible mammalian cell lines expresssing viral proteins was investigated as this would give added flexibility to the CAT rescue system by regulating the levels of SV5 proteins being expressed, but would also allow the development of a parallel project on the examination of molecular interactions between viral proteins.

2 Inducible expression of SV5 proteins in mammalian cell lines

This section deals with the generation, isolation and characterisation of BalbC cell lines which inducibly express SV5 proteins. A modification to the tetracycline Transcriptional Activator (tTA) system is described and subsequently utilised to generate the inducible cell lines. This was work carried out in collaboration with Mr. B.L. Precious (DNA cloning) and Mr. D. Young from this lab. Mr. Young isolated the initial cell lines which were used by me to generate further cell lines which co-expressed 2 or 3 SV5 proteins.

2.1 Overview of expression from tTA system

2.1.1 How does tTA work?

Fig.32 shows a schematic overview of the control of transcription by the tetracycline Transcriptional Activator (tTA) first published by Gossen and Bujard (1992). The tetracycline transcriptional activator is activated and inactivated by the absence or presence of tetracycline repectively, based on the regulatory elements of the tetracycline resistance operon of *E.coli*. tTA was generated by fusion of the Tn-10-derived teracycline repressor (*tet* R) with the activation domain of VP16 from herpes simplex virus (HSV). This binds specifically to its heptamerized operator (*tet* O) which is placed upstream of the human cytomegalovirus (hCMV) minimal promoter and this, in turn, is upstream of the gene of interest. In the absence of tetracycline, *tet* R binds *tet* O and through VP16 interaction with host cell transcription factors and RNA polymerases, the gene of interest is transactivated. In the presence of tetracycline, however, *tet* R binding to *tet* O is blocked and transactivation of the gene of interest is prevented.

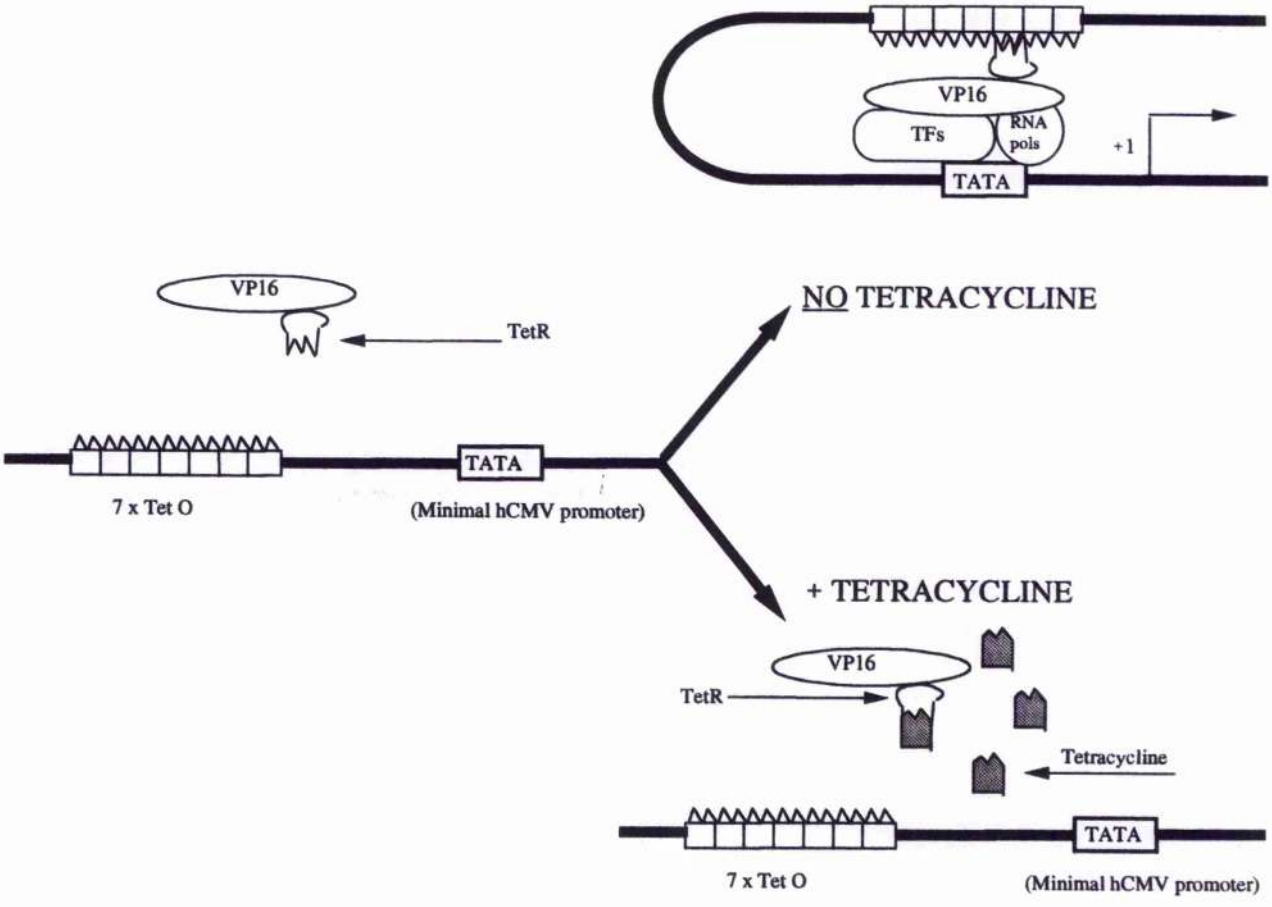
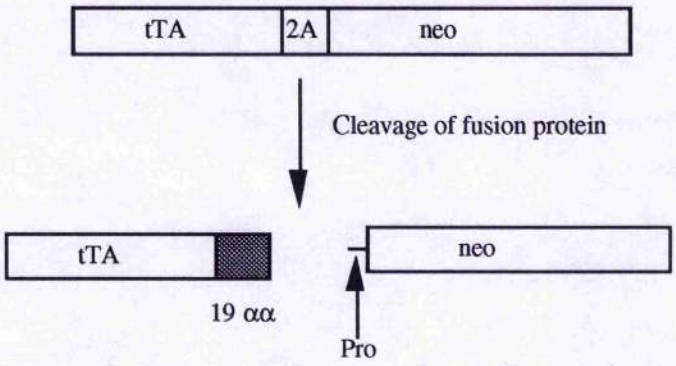


Fig.32:Overview of tTA transcriptional control

Schematic representing the activation and inactivation of tTA by tetracycline based on the regulatory elements of the tetracycline resistance operon of *E.coli*. Fusion of the Tn-*10* -derived tetracycline repressor (*tet*R) with the activation domain of VP16 from HSV generated a transactivator (tTA) which bound specifically to its heptamerized operator (*tet*O). *tet*O was placed upstream of a human cytomegalovirus (hCMV) minimal promoter which in turn was upstream of a gene of interest. In the absence of tetracycline, tTA bound to *tet*O and *trans*activated the gene of interest via VP16 interactions with host cell transcription machinery represented as transcription factors (TFs) and RNA polymerases (RNA pols). The binding of *tet*R to *tet*O is blocked by the addition of tetracycline which itself binds *tet*R thus preventing *trans*activation of the gene of interest. Adapted from Didcock (1996).

To establish cell lines which constitutively expressed tTA in the initial study (Gossen and Bujard, 1992), two plasmids had to be transfected, one encoding tTA and the other conferring resistance by expression of a gene such as neomycin aminoglycoside phosphotransferase (neo), which results in resistance to the neomycin analogue Geneticin (G418; Gibco-BRL). Alternatively, a gene which expressed a resistance phenotype could be cloned into the plasmid encoding tTA (Gossen *et al*, 1994). The level of success with these approaches was found to be variable and many cell lines had to be screened before one which expressed useful levels of tTA was obtained.

Here, an alternative approach was tried in which the gene encoding tTA was fused, via a sequence encoding the Foot-and-Mouth-Disease Virus (FMDV) 2A cleavage peptide (Ryan and Drew,1994), to the neo gene in a single long ORF. Ryan and Drew (1994) demonstrated that cleavage of fusion proteins at the 2A cleavage domain resulted in the addition of 19 amino acids to the C-terminus of the N-terminal domain of a fusion protein (in this case tTA) and a single proline residue to the N-terminus of the C-terminal domain of a fusion protein (neo).



The original tTA driver plasmid, pUHD15.1, contained the hCMV promoter and tTA region but had no selectable marker for the isolation of mammalian transfectants. tTA/neo driver fusion was constructed to increase expression levels of tTA during isolation of transfected cells and introduce a selectable marker (neo) at the same time. The resultant plasmid was called pMR101/tTA and contained a single ORF of tTA/FMDV 2A/neo. Once transfected, cells containing the driver fusion plasmid, pMR101/tTA, were selected by resistance to the neomycin analogue Geneticin (G148). This demonstrated that cleavage of the fusion protein had occurred at the 2A site to

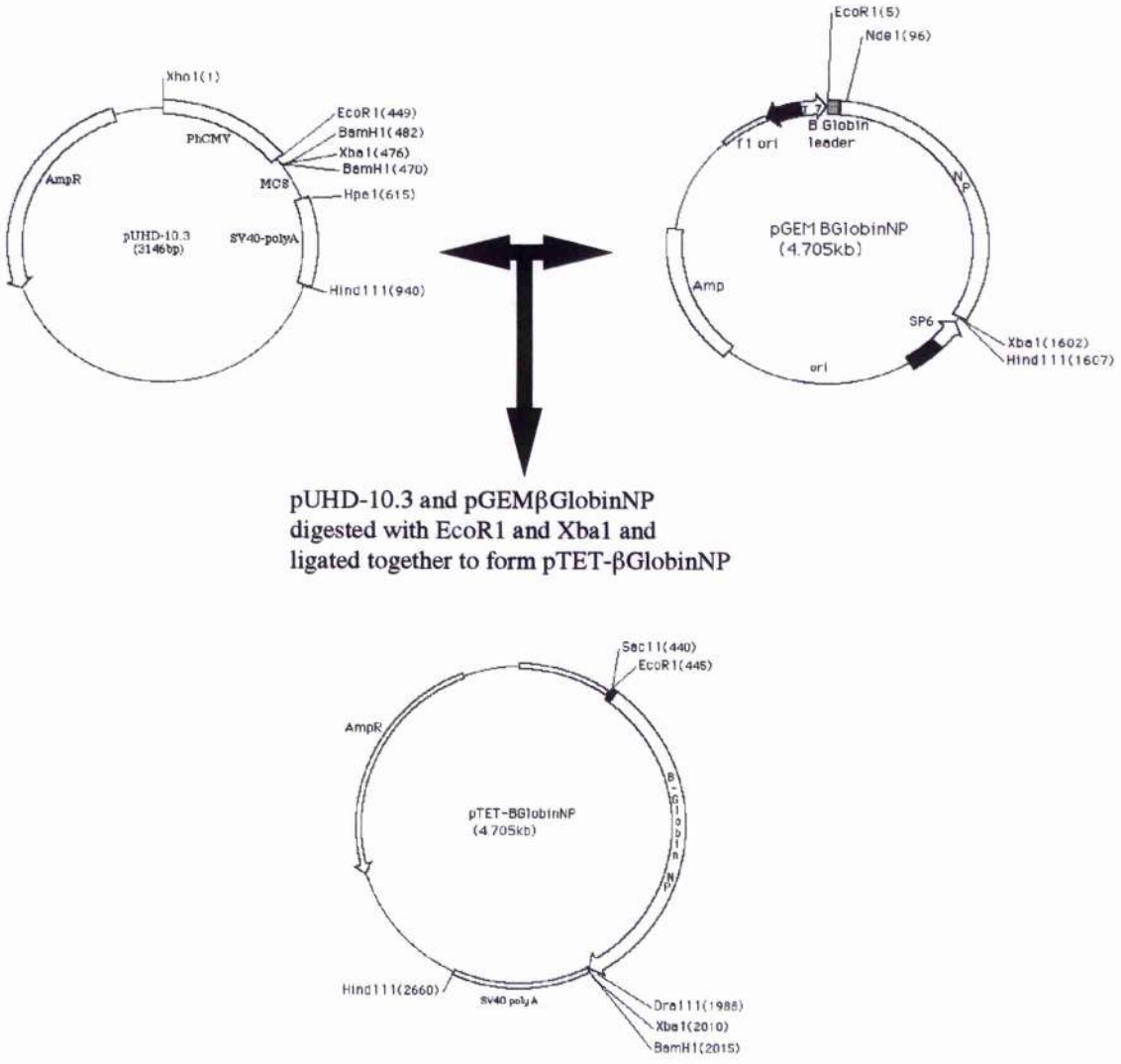


Fig.33:Construction of pTET-βGlobinNP (pTET-NP)

SV5 NP ORF cloned into pUHD-10.3 for inducible expression via the tetracycline-controlled transactivator in mammalian cell lines. The NP ORF including the βGlobin leader sequence was excised from pGEMβGlobinNP by digesting with EcoR1 and Xba1 restriction enzymes. The purified fragment was ligated to pUHD10.3 which had also been digested with EcoR1 and Xba1. The resultant plasmid was called pTET-βGlobinNP.

produce fully functional neo. Therefore, the addition of the proline residue at the N-terminus of neo did not interfere with its function. It was subsequently shown that the 19 amino acid addition at the C-terminus of tTA did not affect its ability to transactivate transcription of the SV5 gene(s) of interest (Precious *et al*, 1995).

2.1.2 Construction of responder plasmids for tTA system

Once tTA is activated (i.e. in the absence of tetracycline), it binds to *tet* O on the responder plasmid(s), thus transactivating the gene(s) of interest (Fig.32). The construction of the pTET responder plasmids was carried out in collaboration with Mr. B.L. Precious who made pTET-P and pTET-V. Fig.33 shows a schematic diagram of the construction of pTET-βGlobinNP (or pTET-NP) from pGEM-βGlobinNP. The NP ORF, including the β-globin enhancer region, was excised from pGEM-βGlobinNP by digestion with EcoR1 and Xba1 restriction enzymes and purified by gel extraction. The NP fragment was cloned into the EcoR1/Xba1 sites of pUHD10.3 and the resultant plasmid was called pTET-NP. All 3 plamids, pTET-NP, pTET-P and pTET-V, were used as the responder plasmids for activation by tTA.

2.2 Generation of cell lines expressing SV5 proteins

BalbC cells were transfected with the tTA/neo driver fusion plasmid and the appropriate responder plasmid (pTET-NP, pTET-P or pTET-V), to generate the cell line clones as described in Materials and Methods, Chapter 2, Section 4. Once positive clones had been identified by immunofluorescence and verified by western blot, the clones with the highest number of positive cells were sub-cloned. This aimed to enrich for cells expressing high levels of the protein of interest by expanding colonies from single cells (Described in Materials and Methods, Chapter 2, Section 4.4). A subclone from each cell type showing the highest level of protein expression, as judged by immunofluorescence, was chosen for further analysis. The cell lines expressing NP, P

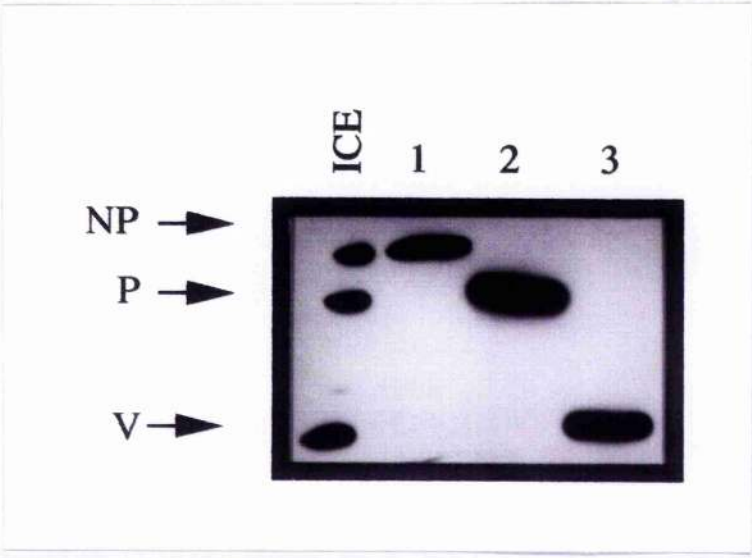


Fig.34 : Western blot of NP, P and V cell line extracts.
Cell lines expressing NP, P or V proteins were induced for 36 hours by incubating the cell lines in the absence of tetracycline. The cells were washed in PBS and harvested by scraping into ice-cold PBS and stored in 1 ml aliquots (See Materials and Methods, Chapter 2, Section 5.14 for details). A sample of each extract was added to SDS-lysis buffer and boiled for 2 mins before loading onto a 10% SDS-slab gel for separation of the polypeptides by electrophoresis. In the resultant western blot, NP (Lane 1), P (Lane 2) and V (Lane 3) cell extracts demonstrated that full-length authentic viral proteins were expressed since the proteins of interest were similar in size to the native proteins from and infected cell extract (ICE). The P and V proteins were detected with mAb SV5 P-k and the NP protein was detected with SV5 P-d.

or V were generated and isolated by Mr. D. Young from this lab and are included here for completeness.

2.2.1 BalbC cell lines expressing NP, P or V

Once isolated, the protein expression from each cell line was assayed by western blot to verify full length authentic proteins were being expressed. This was demonstrated in Fig.34 Lanes 1, 2 and 3 where NP, P and V proteins from the respective cell lines were shown to be similar in size to the native viral proteins from an infected cell extract (ICE). Protein expression from all 3 cell lines was subsequently shown to be under tTA control since the levels of protein expression were seen to increase significantly upon induction (Precious *et al*, 1995).

Intracellular localization of the NP, P and V proteins was then examined by immunofluorescence and confocal microscopy, revealing distinct differences in the distribution patterns of NP, P and V proteins when expressed individually. P protein had a generally diffuse cytoplasmic distribution but some fine filamentous structures could be seen. In addition, the majority of cells contained a single focal point (occasionally two) of accumulated P fluorescence close to or on the nuclear membrane (Fig.35, Panel a). In contrast to this, V protein had a diffuse, primarily nuclear distribution, but could be visualised in the cytoplasm when expressed at high levels (Fig.35, Panel b). The NP protein was distributed throughout the cytoplasm where it was visualised as punctate and granular fluorescent loci (Fig.35, Panel c).

2.2.2 Isolation of cell lines expressing P+NP and V+NP

To examine (potential) viral protein:protein interactions, cell lines co-expressing NP+P and NP+V were generated by co-transfecting the driver fusion plasmid with the appropriate pTET responder plasmids. The resultant colonies were screened for protein expression as described above and a clone from each cell type with the highest level of protein expression, was analysed further.

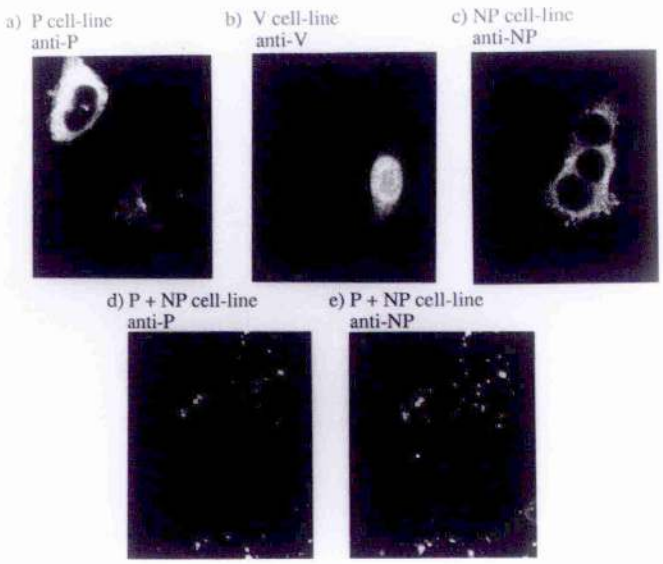


Fig.35 : Immunofluorescence of NP, P, V and NP+P cell lines.
Photgraphs illustrating the intracellular distibition of P, V and NP in cells which had been released from a tetracycline block for 24 hours and were expressing the P, V, NP or NP+P proteins, as visualised by confocal microscopy. Monolayers were fixed and stained by direct fluorescence using FITC-labelled SV5 P-k, which detects both P and V proteins, and rhodamine-labelled SV5 NP-a, which detects NP. Cells expressing both NP + P were stained simultaneously with both antibodies.
Figure taken from Precious *et al*, 1995.

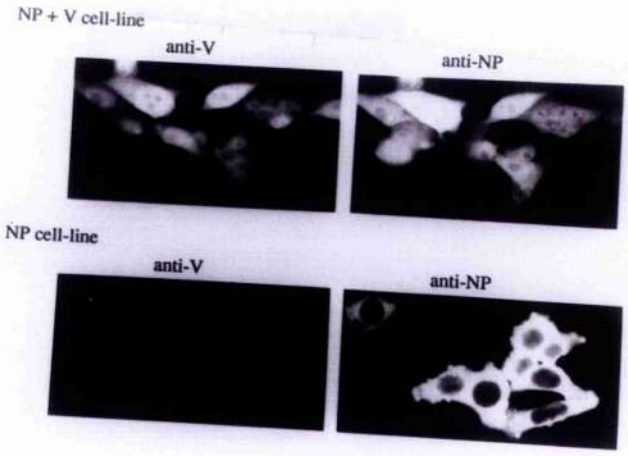


Fig.36 : Immunofluorescence of NP+V and NP cell lines.
Photographs comparing the distributions of the NP protein in NP and NP+V expressing cell lines. Monolayers were released forom the tetracycline block for 24 hours, fixed and stained by direct fluorescence with FITC-labelled SV5- P-k to detect V, and rhodamine-labelled SV5 NP-a to detect NP. Figure taken from Precious *et al*, 1995.

The intracellular distribution of the co-expressed proteins was examined by immunofluorescence. Fig.35-d shows that co-expression of the NP and P proteins led to the formation of large cytoplasmic aggregates. These consisted of co-localised NP+P proteins in inclusion bodies similar to those seen at late times in an SV5 infection. This was very different to the fluorescence patterns seen when the proteins were expressed alone. (Compare Panels a and c with d and e in Fig 35)

However, when co-expressed with V, NP was partially redistributed from the punctate granular fluorescent loci, seen when it was expressed alone, (Fig 35 panel c and Fig 36, bottom right panel), to diffuse cytoplasmic and nuclear fluorescence (Fig 36, top right). Furthermore, no NP-V inclusion body-type aggregates could be detected.

2.2.3 Further isolation of NP+V cell lines

In the initial NP+V cell line, the number of cells co-expressing NP and V proteins was very low and this number was not substantially increased by subcloning. Therefore, a new NP+V cell line was generated by transfecting the V expressing cell line with pTET-NP and a plasmid containing a selectable marker. The V cell line had been shown to produce high levels of tTA which could be utilised to drive NP simultaneous to V expression. The V expressing cell line was therefore co-transfected with pTET-NP and pREP4 (Invitrogen) which contained the hygromycin resistance gene, and subsequently selected by resistance to Hygromycin B. From 48 initial clones screened, 4 were positive for NP expression and were all subcloned to enrich for NP expressing cells. From a total of 96 subclones, only 1 expressed NP but, unfortunately, in this clone, V expression had been lost (Data not shown).

A second attempt at generating NP+V cell lines was then made by transfecting naive BalbC cells with the driver fusion plasmid and the responder plasmids as described previously. From 48 clones, 3 were positive for NP and V expression and the one expressing highest levels of protein was analysed further.

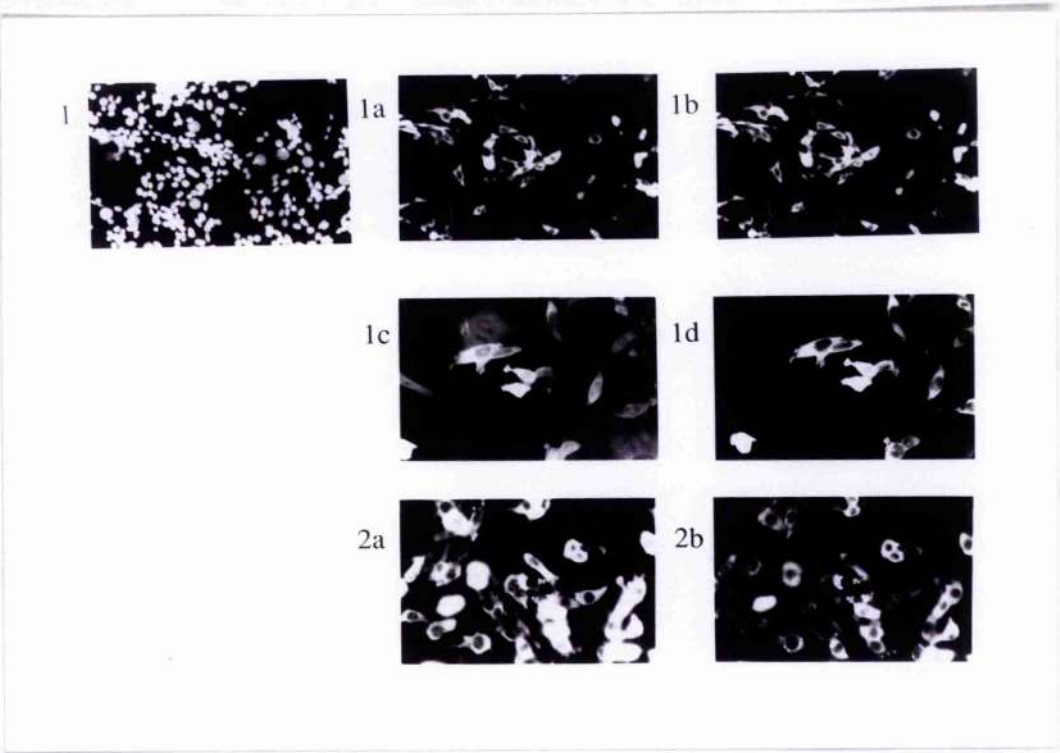


Fig.37 : Immunofluorescence of NP+V and NP+P+V cell lines.

Photographs illustrating the distribution patterns of NP and V proteins in the new NP+V cell line (Panels 1a, b, c and d) and the distribution of NP and P proteins in the NP+P+V cell line (Panels 2a and 2b). Both cell lines were released from a tetracycline block for 36 hours, then fixed and simultaneously stained with DAPI (Panel 1) and by direct fluorescence with FITC-labelled SV5 P-k to detect V (in NP+V cell line, Panels 1a and 1c), FITC-labelled SV5 P-e to detect P (in NP+P+V cell line, Panel 2a) and rhodamine-labelled SV5 NP-a to detect NP (Panels 1b, 1d and 2b).

Fig.37 Panels 1a, b, c and d show that the intracellular distributions of the NP and V proteins were identical to that seen with the original NP+V cell line (compare to Fig.36) but the percentage of expressing cells had increased. Therefore, when NP and V proteins were co-expressed, NP was again redistributed to give diffuse cytoplasmic and nuclear fluorescence.

Fig.38 shows a time course of release from the tetracycline block (induction of protein expression) from cell lines expressing NP, NP+V and NP+P proteins, and analysed by western blot. This demonstrated that each cell line expressed full length authentic proteins with optimum protein accumulation at 60 hours post-tetracycline release.

2.2.4 BalbC cell lines co-expressing V+P and V+NP+P

To investigate potential P+V interactions, a cell line expressing both proteins was generated and examined by immunofluorescence for any change in P or V distribution compared to when expressed alone. The cell line was generated by transfecting the V cell line with pTET-P plus pREP4 as described in 2.2.3. From 24 initial colonies of the V+P cell line, there were 5 positive clones, the best of which was subcloned and screened by western blot. Fig.39 shows a western blot of the first 8 subclones. As expected, the majority of the subclones expressed V protein but only subclone number 2 (Lane 2) was positive for the prescence of P.

The P+V cell line was then examined by immunofluorescence where no change in the distribution of either protein was seen, suggesting there was no discernible P:V interaction detected by this method.

A cell line expressing V+NP+P was generated as described in 2.2.3, the aim being to supply SV5 proteins necessary to encapsidate a le-CAT-tr negative sense RNA transcript for CAT rescue. pTET-NP and pTET-P were transfected into the V expressing cell line as described in 2.2.3. The resultant clones were initially screened for NP and P expression by IF. From 24 initial clones of the V+NP+P cell line, 4 were positive for NP and P fluorescence, the best of which is shown in Fig.37 Panels 2a and

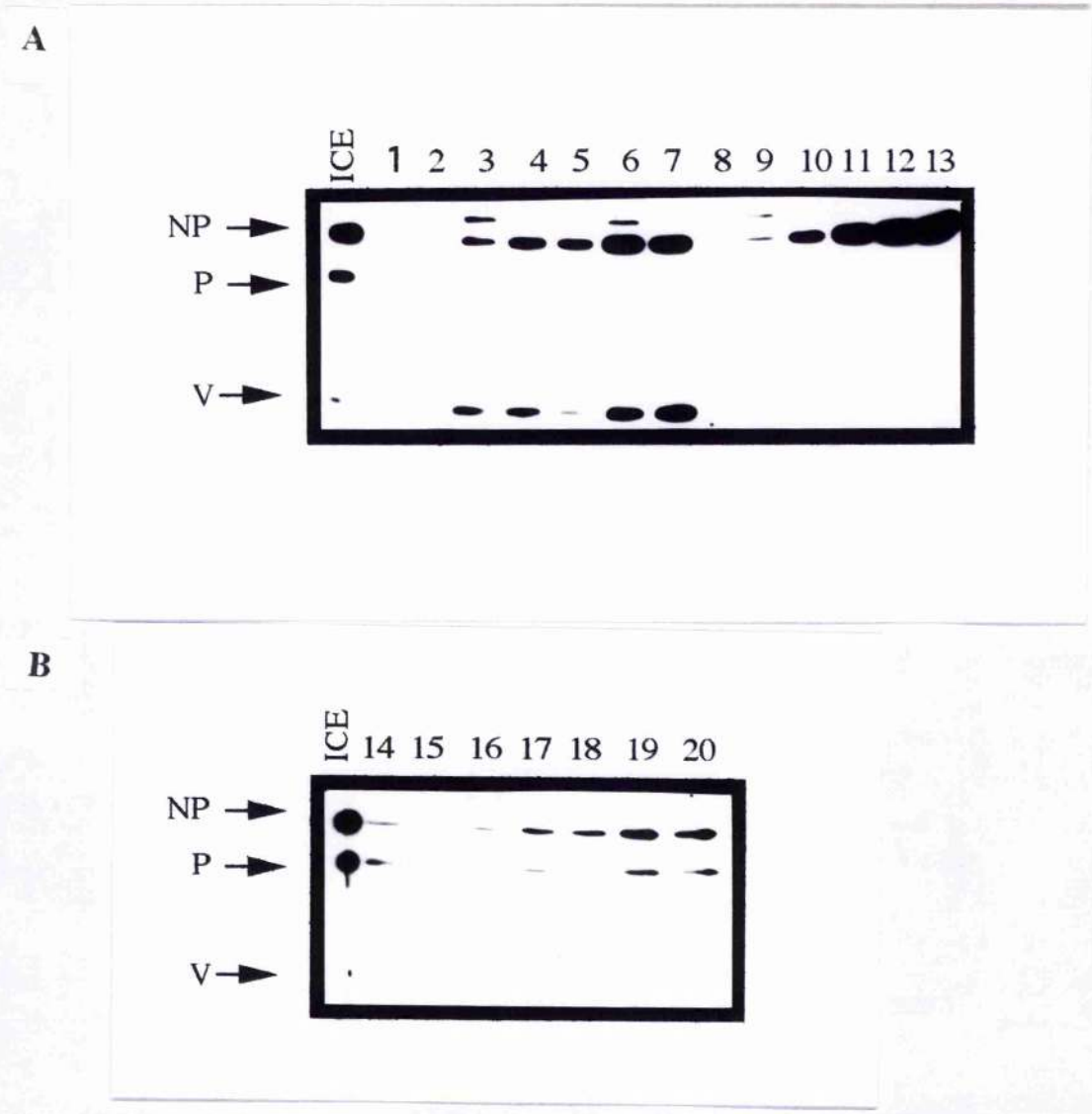


Fig.38:Time course of induction for BalbC NP+V, NP and NP+P cell lines

Western blot of time course of induction from BalbC NP+V, NP (Panel A) and NP+P cell lines (Panel B). Cells were plated into 6 well dishes and induced for between 12 and 72 hours, harvested in 500 µl PBS, sonicated and spun at 14,000 rpm in a cold benchtop microfuge. 30 µl of the resultant supernatant was added to 30 µl of lysis buffer, boiled and 20 µl loaded on a 10% gel for SDS-PAGE and western blotting. Panel A, Lanes 1-7 represent BalbC NP+V cells induced for 0, 12, 24, 36, 48, 60 and 72 hours respectively. Lanes 8-13 represent BalbC NP cells induced for 0, 12, 24, 48, 60 and 72 hours respectively. Panel B Lanes 14-20 represent BalbC NP+P cells induced for 0, 12, 24, 36, 48, 60 and 72 hours respectively. The polypeptides were detected with mAbs SV5-NPd and SV5-Pk and the antibodies were detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. An SV5 infected cell extract (ICE) was also included to show where the NP, P and V polypeptides migrated in the polyacrylamide gel.

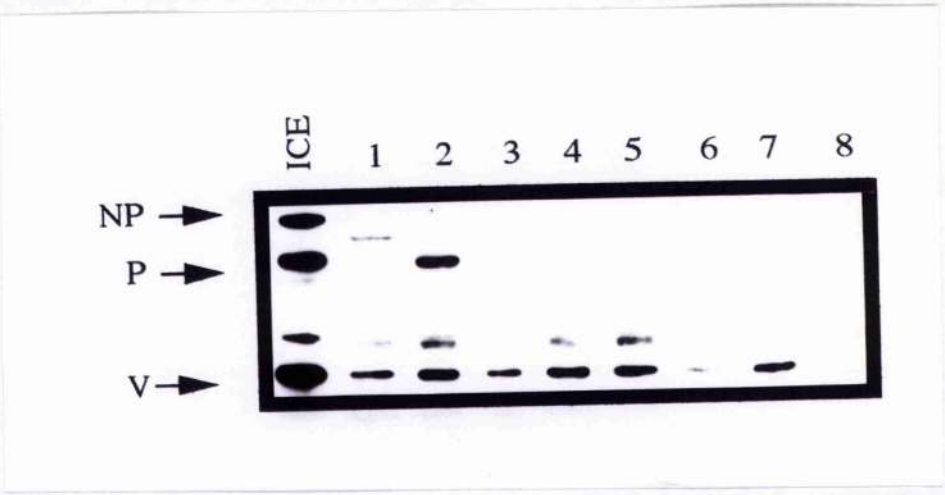


Fig.39:Protein expression from BalbC V+P sub-clones

BalbC cell line sub-clones were screened by western blot for SV5 V+P protein expression. Each sub-clone was grown in one well of a 24 well dish and induced for 48 hours. Cells were lysed in 1 ml of lysis buffer, sonicated and 20 µl loaded onto a 10% polyacrylamide gel for SDS-PAGE and western blotting. BalbC V+P sub-clones 1-8 are presented here as indicated above each lane. Polypeptides were detected using mAbs SV5-Pk and SV5-NPd The bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. An SV5 infected cell extract (ICE) was also included to show where the NP, P and V polypeptides migrated in the polyacrylamide gel.

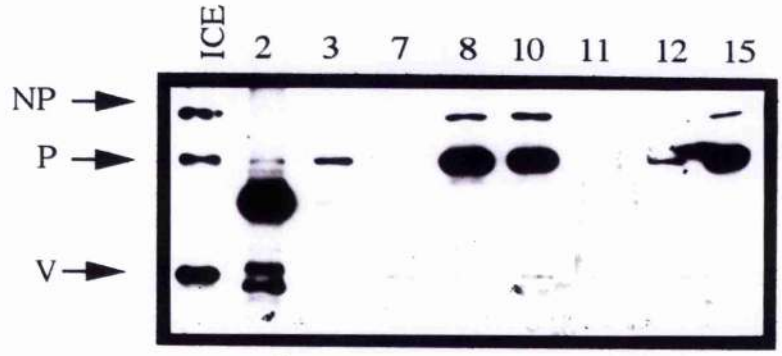


Fig.40:Protein expression from BalbC NP+P+V cell line sub-clones

BalbC cell line sub-clones were screened by western blot for SV5 NP+P+V protein expression. Each sub-clone was grown in one well of a 24 well tissue culture dish, induced for 36 hours and harvested in 400 µl of lysis buffer. Cells were sonicated, boiled and 20 µl of each sample was loaded onto a 10% polyacrylamide gel for SDS-PAGE and western blotting. The numbers above each lane correspond to the clone number analysed (i.e. BalbC NP+P+V sub-clones 2, 3, 7, 8, 10, 11, 12 and 15 are presented here) The polypeptides were detected with mAbs SV5-NPd and SV5-Pk. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. An SV5 infected cell extract (ICE) was also included to show where the NP, P and V polypeptides migrated in the polyacrylamide gel.

2b where NP+P cytoplasmic aggregates were clearly visible. This clone was subcloned and screened by western blot to assay for the presence of all three proteins. Fig.40 shows a western blot from V+NP+P subclones. Although 3 subclones clearly expressed both NP and P proteins (Lanes 8, 10 and 15) they had lost their ability to express V. Since previous fluorescence data from NP+V cells had suggested a role for V in keeping NP from self-aggregating, and that NP+P formed large inclusion body-type aggregates, one could predict that the absence of V in a V+NP+P cell line would lead to formation of cytoplasmic aggregates similar to those seen in NP+P cells. This could account for the NP+P aggregates seen in the fluorescence of the initial clone. However, some diffuse NP fluorescence was seen suggesting that significantly more soluble NP was produced in the V+NP+P cell line than in the NP+P cell line. Therefore, the V+NP+P cell line was used to supply the encapsidation proteins for the CAT rescue system, with any auxiliary proteins necessary for transcription and replication being supplied by SV5 helper virus.

2.3 Uses for the inducible cell lines

2.3.1 CAT rescue in V+NP+P cells

The V+NP+P cell line was used as a noncytocidal alternative to the VacT7 system for providing the viral proteins necessary for the reverse genetics experiments. As previously indicated, the V+NP+P cell line produced very little V protein but did contain a significant amount of soluble NP, as judged by a diffuse immunofluorescence pattern. It was therefore hoped that this cell line would be able to supply the proteins necessary to encapsidate a synthetic negative sense transcript for CAT rescue. The auxiliary proteins necessary for transcription and replication of the encapsidated transcript were supplied by SV5 helper virus.

At 24 hours post-tetracycline release, the cells were transfected with RNA from pT7.1 and pPanHan, as described in 1.4.3. The cells were infected with SV5 at 12 hours post-transfection and harvested for CAT assay 24 hours post-infection. No CAT activity was detected using RNA from either pT7.1 or pPanHan (Data not shown).

Although the results from the reverse genetics system were disappointing, a number of valuble reagents were generated, with the inducible cell lines being the most useful.

2.3.2 Analysis of NP-P and NP-V complexes by CsCl gradients

Immunofluorescence data from NP+P and NP+V cell lines had demonstrated a difference in NP distribution from that seen when NP was expressed alone and suggested that NP+P complexes were insoluble while NP+V complexes were soluble. An attempt was made to demonstrate the differences in density of the NP:P and NP:V complexes by caesium chloride (CsCl) density centrifugation. It was hoped that soluble NP:V complexes would be found at the top of the gradient, while insoluble complexes would be found at the bottom. Unfortunately, both the NP+P and NP+V complexes completely dissociated in the presence of CsCl (Data not shown), and so another way of examining these interactions was necessary.

2.4 Conclusions from the Generation of Inducible Cell Lines

The results using the cell lines for the reverse genetics system were again disappointing, but not altogether surprising. The cell lines proved to be good reagents with which to examine the the interactions of the SV5 proteins involved in the control of viral encapsidation, transcription and replication.

When co-expressed with NP, P seems to bind to NP aggregates resulting in larger cytoplasmic aggregates than those seen when NP is expressed alone. However, the change of the intracellular distribution of NP when co-expressed with V (compared to when expressed alone), suggests a possible role for V in keeping NP soluble prior to an ordered encapsidation process. Therefore, the focus of the project moved towards elucidating the viral protein : protein interactions within the replication complex, and thereby understand more clearly the the roles played by these proteins in the viral transcription / replication mechanism.

3 SV5 Protein : Protein Interactions

Generation of the cell lines had demonstrated by immunofluorescence that NP could bind both P and V proteins. However, since examination of the NP:P and NP:V interactions had been unsuccessful using CsCl density centrifugation, another approach for the investigation of these interactions was devised.

This section describes the development of a solid phase panning assay (Capture Assay) which utilised extracts from the NP, P and V expressing cell lines. The assay was used to examine direct NP:P and NP:V interactions. The differences between NP:P and NP:V interactions were investigated and the binding site on P for polymeric NP was examined by C-terminal deletion mutant analysis. Therefore, the results presented in this section give some insight into the protein : protein interactions taking place within the replication complex and suggest possible roles for these proteins in SV5 transcription / replication mechanisms.

3.1 NP-P and NP-V interactions examined by capture assay

NP:V interactions had been shown to be sensitive to both ionic and non-ionic detergents (Precious *et al*, 1995) and as a consequence, simple co-immunoprecipitations of mixed supernatant extracts from cells expresing NP, P or V proteins were not successful. In an alternative approach, a novel protein : protein capture assay was developed by Dr. R.E. Randall in this laboratory, and used by me to investigate direct NP:P and NP:V interactions.

3.1.1 Overview of capture assay

It had been previously shown that fixed and killed *Staphylococcus aureus* bound strongly to certain kinds of plastic (Randall, 1983), and this property was used as the basis for the solid phase panning assay. Full details are given in Materials and Methods

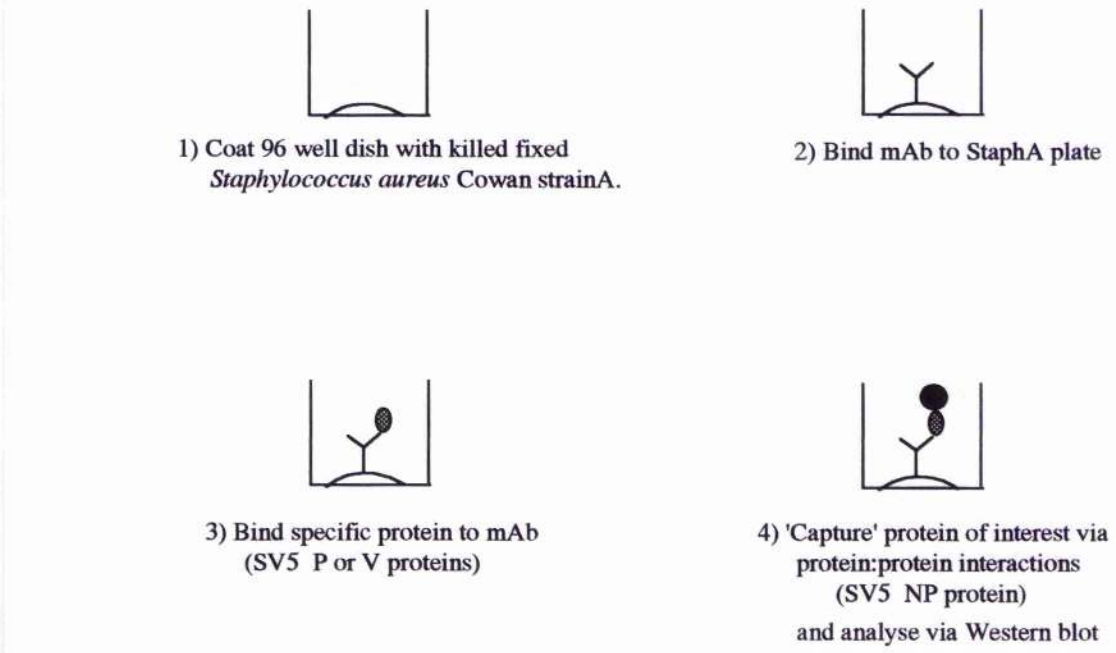


Fig.41:Protein : protein capture assay.

96 well microtitre plate flooded with 0.2% suspension of fixed and killed *Staphylococcus aureus* Cowan strain A (*S.aureus*) in PBS and left for a minimum of 4 hours at 4 ^{O}C. Unbound material was removed by washing with PBS, leaving a confluent monolayer of *S.aureus* bound to the plate. This monolayer was cross-linked to the surface of the plastic by incubating the plate in a PBS solution containing 5% (v/v) formaldehyde and 2% (w/v) sucrose for 10 mins at RT. The plate was washed extensively in PBS whereupon monoclonal antibodies (mAbs) were bound to the monolayers by adding 50 µl of mAb per well and incubating at 4 ^{O}C for 2 hours. Unbound mAb was removed by washing with PBS and any non-specific protein binding sites were blocked by addition of 10% bovine serum albumin (BSA) for 1 hour at 4 ^{O}C. The P and V proteins were captured by the mAbs by adding 100 µl (per well) of extracts from the cells expressing these proteins, and incubated at 4 ^{O}C for 2 hours with continuous rocking. Unbound material was removed by washing 4 times with PBS. To examine the binding of the NP protein to the captured P or V proteins, 100 µl of extract from cells expressing the NP protein were incubated with the appropriate wells for 2 hours at 4 ^{O}C with continuous agitation. Unbound material was removed by washing 4 times with PBS. 30 µl of disruption buffer added to each well, the plate was heated to 100 ^{O}C for 5 mins and samples run on SDS-PAGE and analysed by Western blot.

Chapter 2, Section 5.13 but briefly, a 96 well micotitre plate was coated with a fixed killed suspension of *S.aureus* . A mAb specific to the amino-cotermini of the P and V proteins was then bound to the immuno-absorbant coating on each well. Extracts from the P and V cell lines were then 'captured' by the mAb and the ability of the captured proteins to bind NP was examined by western blot analysis. A schematic diagram of the capture assay is given in Fig.41.

3.1.2 Direct interaction of NP with both P and V

The mAb, SV5 P-k, which recognises an amino-terminal epitope common to both P and V proteins, was added to *S. aureus* coated wells of a 96 well microtitre plate (StaphA plate)and incubated for 2 hours at 4 °C with shaking. Non-specific protein binding sites were blocked by the addition of blotto (10% (w/v) skimmed milk powder in PBS) for 1 hour at 4 °C with shaking. Extracts from the BalbC P and V expressing cell lines were prepared by harvesting the cells in PBS whereupon the cells were then sonicated and the cell debris pelleted by centrifugation (See Materials and Methods, Chapter 2, Section 5.14 for details). The supernatants were decanted in preparation for addition to the StaphA plates . 100 µl of either P or V extract was added to a well for immobilisation by incubating the plate at 4 °C with shaking for 1-2 hours. Any unbound material was removed by washing extensively in PBS.

A low speed supernatant (LSS) extract was then prepared from NP cells by harvesting the cells in PBS, sonicating them and then removing cell debris by centrifugation at 6,500 rpm on a benchtop microfuge (See Materials and Methods, Chapter 2, Section 5.14 for details). 100 µl of the NP LSS was added to the wells containing immobilised or 'captured' P or V proteins. NP was incubated with P or V for 2 hours at 4 °C with shaking and unbound material was again removed by washing extensivly in PBS. Direct protein : protein interactions were analysed by adding 30 µl of SDS-lysis buffer to each well whereupon the plate was boiled for 5 mins. Each sample was

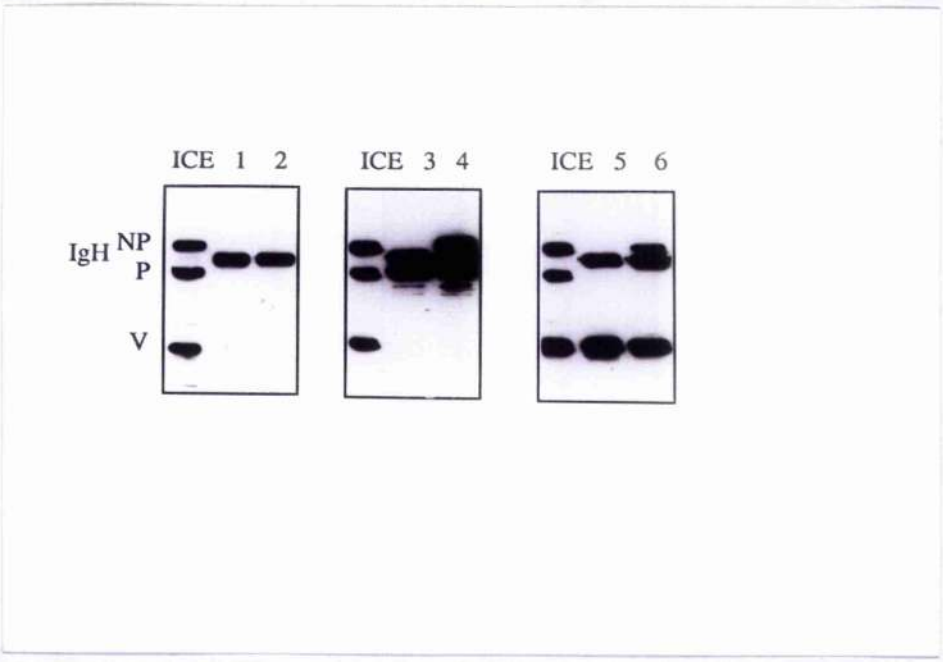


Fig.42 : NP can bind to both P and V proteins.

Protein:protein capture assay demonstrating NP:P and NP:V interactions. The mAb SV5 P-k was bound to wells of a *S.aureus* microtitre plate (Lanes 1-6). Low speed supernatant extracts of cells expressing P, V or NP were made. The P extract was added to wells 3 and 4, while the V extract was added to wells 5 and 6, and PBS was added to wells 1 and 2. Following binding of P and V to the mAb SV5 P-k, the wells were thoroughly washed with PBS and the NP extract was added to wells 2, 4 and 6. The NP extract was incubated with P or V for 2 hours at +4 °C, whereupon the wells were washed again in PBS. The residual polypeptides were seperated by electrophoresis on a 10% SDS-polyacrylamide slab gel and visualised by western blot analysis. P and V proteins were detected with the mAb SV5 P-k and NP was detected with the mAb SV5 NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep-anti-mouse Ig. The anti-mouse Ig also detected the heavy chain (IgH) of the immobilising antibody (SV5 P-K) which was bound to the *S.aureus* and was thus visualised in the western blot. An infected cell extract (ICE) was also western blotted to show the positions to which the NP, P and V proteins migrated in the polyacrylamide gel.
Figure taken from Randall and Bermingham, 1996.

electrophoresed through a 10% SDS-polyacrylamide gel for western blot analysis(See Materials and Methods, Chapter 2, Section 5.5 and 5.6 for details).

A direct interaction between NP and both P and V proteins is shown in Fig.42 (Lanes 4 and 6). This interaction was only seen in wells containing P or V protein (Lanes 4 and 6) and not in the control well containing mAb alone (Lane 2). Furthermore, the amount of NP bound by V was significantly less than that bound by P (Compare Lanes 4 and 6), suggesting that V could only bind a subset of the NP recognised by P.

3.1.3 V binds a subset of the available NP

The results described above suggested that V could only bind a subset of the NP available for binding and which is recognised by P. This was further tested in an absorption experiment on a StaphA plate coated with SV5 P-k. A series of wells containing either P or V proteins was prepared as described above. 100 µl of a LSS extract from the NP expressing cells was added to the first well containing V and incubated for 1 hour at 4 °C with shaking. The unbound material from the V containing well was then transferred to the adjacent V-containing well and incubated for a further hour. (The bound polypeptides from the first well were harvested in SDS-lysis buffer for electrophoresis and western blot analysis.) The absorption step (serial passage of NP extract across V-containing wells) was repeated 5 times and the residual fraction was tested for its ability to bind P by adding it to a well containing P protein. The serial passage of NP extract across V wells is shown in Fig.43 Panel b, Lanes 10-15. NP binding to V could be seen in Lane 10 but in none of the subsequent wells, demonstrating the small amount of NP initially bound by V had been absorbed out of the NP extract. The unbound material from Panel b, Lane 15, was then added to a well containing P (Panel a, Lane 8) and showed that the pre-absorbed extract still contained NP which had the ability to be bound by P.

The experiment was also done in reverse where NP was bound to a series of P wells (Panel a, Lanes 2-7) whereupon the residual material was assayed for its ability to bind

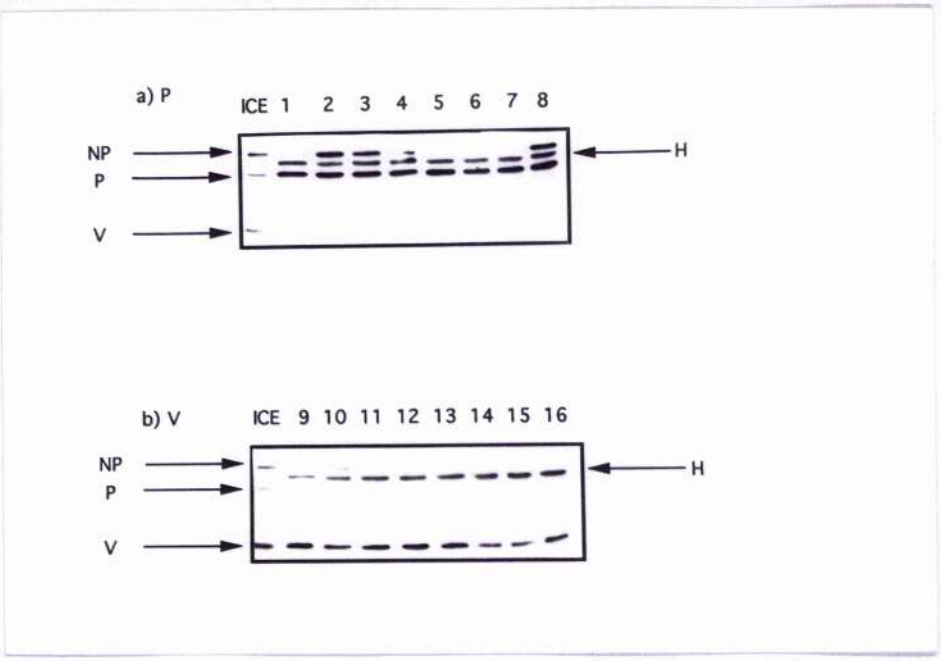


Fig.43 : V binds a subpopulation of the available NP.

Western blot analysis demonstrating that preabsorption of NP with P removed the fraction of NP that bound V, but preabsorption of NP with V, did not remove all the NP that bound to P. The mAb SV5 P-k, attached to *S.aureus* microtitre plates, was used to capture P (Panel a) or V (Panel b) from low speed supernatant extracts of cells expressing these proteins. A low speed supernatant extract of NP-expressing cells was added for 2 hours at 4 °C, to wells 2 and 10. The unbound material from these wells was collected and incubated with P and V present in 3 and 11 respectively. Similar absorptions of the NP protein continued until wells 7 and 15 respectively. Following this final absorption, the unbound material which had been incubated with P was added to a well containing V (Well 16), and the unbound material which had been incubated with V was added to a P containing well (Well 8). Polypeptides in all the wells were seperated by electrophoresis through a 10% SDS-polyacrylamide gel and analysed by western blot. The Pand V proteins were detected with the mAb SV5 P-k and the NP protein with the mAb SV5 NP-d. An infected cell extract (ICE) was included to show the positions to which the native NP, P and V polypeptides migrated in the polyacrylamide gel.
Figure taken from Randall and Bermingham, 1996.

V. However, after NP was preabsorbed with P, there was no NP available to bind V (Panel b, Lane 16).

These results were consistant with the hypothesis that V could only bind a subpopulation of NP recognised by P. After a series of preincubation steps with V, a substantial amount of NP remained in the unbound material, which could be subsequently bound by P. (Panel a, Lane 8) This, however, was not the case in reverse. After preincubation with P, there was no NP available to be bound by V. Furthermore, the results suggested there was a difference in NP populations; one recognised by P alone and the other recognised by both P and V.

3.1.4 P binds soluble and polymeric NP while V binds only soluble NP

To further elucidate the differences in NP populations, the low speed supernatant (LSS) from NP expressing cells was further fractionated by centrifugation at 400,000 x *g* in a benchtop ultracentrifuge (Beckman) for 1 hour at 4 °C. The supernatant was decanted and called "high speed supernatant" (HSS), and the pellet was resuspended in its original volume of PBS by sonication and called "high speed pellet" (HSP). Analysis of these fractions by western blot is shown in Fig.44, Panel a. The majority of the NP from the LSS (Lane 1) was pelleted upon centrifugation (Lane 3) demonstrating that it was polymeric. However, a small fraction remained soluble and was detected in the HSS (Lane 2).

The ability of both P and V to bind to the fractionated NP extracts was then tested. A StaphA plate, containing P or V proteins bound by SV5 P-k, was prepared as described previously. 100 µl of the NP LSS, HSS or HSP extracts were then incubated with the appropriate P or V wells, and assayed by western blot as shown in Fig.44. Panel c (Lanes 7 and 8) clearly demonstrated that P could bind both soluble and polymeric forms of NP. However, Panel d, (Lanes 11 and 12) demonstrated that V could only bind soluble NP (Lane 11) and not polymeric NP (Lane 12).

Again, V bound much less NP from the LSS extract than did P (Compare Lane 7 with Lane 10), but this could now be explained. The LSS extract was a combination of

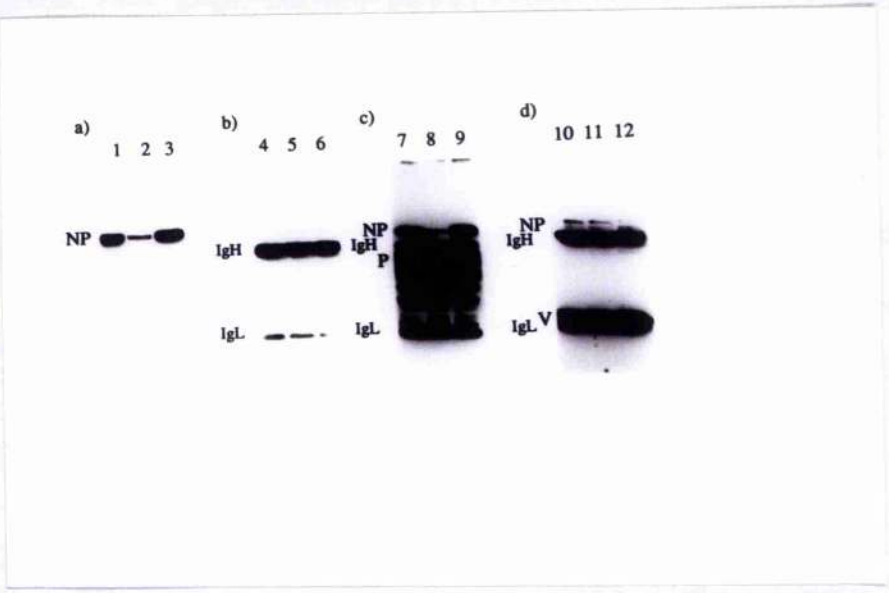


Fig.44 : P binds both soluble and polymeric NP while V binds only soluble NP.

Western blot analysis demonstrating the interaction of the P and V proteins with soluble and polymeric forms of NP. The mAb SV5 P-k was bound to all wells of a *S.aureus* microtitre plate (b, c and d). PBS, P or V proteins were incubated with all the wells of b, c and d respectively. Low speed supernatant (LSS), high speed supernatant (HSS) and high speed pellet (HSP) extracts of cells expressing the NP protein (Panel a, Lanes 1, 2 and 3 rspectively) were then added to wells 1, 2 and 3 respectively to each of b, c and d. Unbound material was removed and the residual polypeptides were seperated by electrophoresis through a 10% SDS-polyacrylamide slab gel, and analysed by western blot as described previously.
Figure taken from Randall and Bermingham, 1996.

soluble and polymeric NP which could all be bound by P. Since V could only bind soluble NP, the small amount of NP bound by V from the LSS, corresponded to the amount of soluble NP in this fraction.

3.1.5 mAb SV5 P-e blocks P binding to polymeric NP

Since the P and V proteins are amino-coterminal, and only P could bind polymeric NP, this suggested that the unique C-terminus of P was site of interaction with polymeric NP. A panel of mAbs had been generated to the P unique region (Randall *et al*, 1987) and were used to determine whether any could block the P interaction with NP. StaphA plates were prepared and coated with each of the mAbs specific to the C-terminus of the P protein. LSS extracts of P expressing cells was bound to the various mAbs and then incubated with LSS, HSS and HSP extracts from NP expressing cells as described above. The bound polypeptides were electrophoresed through a 10% SDS-polyacrylamide gel and analysed by western blot.

Most of the mAbs gave the same binding pattern to that seen with SV5 P-k (Fig.44) indicating that the binding of P to polymeric NP had not been blocked. However, when P was immobilised by mAb SV5 P-e, a change in the binding pattern was observed (Fig.45, Panel b). P could now only bind a small proportion of NP from the LSS as compared to when immobilised by SV5 P-k (Compare Lanes 2 in Panels a and b) and this small amount of NP bound by P corresponded to the soluble NP fraction (Panel b, Lane 3). P could no longer bind polymeric NP (Panel b, Lane 4) and therefore gave a similar binding profile to that seen with V. This suggested that the site of interaction between P and polymeric NP lay, at least in part, within the SV5 P-e recognition sequence.

3.2 Mapping of the mAb SV5 P-e epitope

The binding sites of the P unique mAbs had never been identified and so it was hoped that by mapping the epitope corresponding to the SV5 P-e, the site of interaction

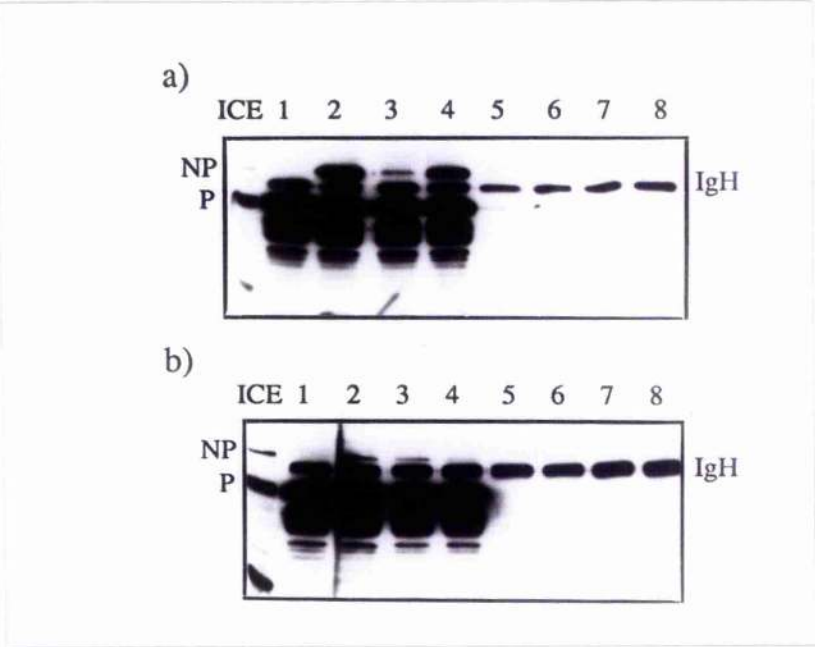


Fig.45 : mAb SV5 P-e blocks P binding to polymeric NP.

Western blot analysis demonstrating the ability of the mAb SV5 P-e to prevent P binding to polymeric NP. The mAbs SV5 P-k (Panel a) or SV5 P-e (Panel b) were immobilised on a *S.aureus* microtitre plate. A low speed supernatant extract of cells expressing the P protein or PBS was added to wells 1-4 and 5-8 respectively. Unbound material was removed by extensive washing in PBS. LSS, HSS and HSP extracts of NP-expressing cells were prepared. LSS was added to wells 2 and 6, HSS was added to wells 3 and 7, and HSP was added to wells 4 and 8 of Panels a and b respectively. PBS was added to wells 1 and 5. Polypeptides, including those of an infected cell extract (ICE) were separated by electrophoresis through a 10% SDS polyacrylamide slab gel for western blot analysis as described previously. Note that the multiple bands visualised with molecular weights lower that P, were degredation products of P as they were recognised by SV5 P-k when the blot was stripped and reprobed using SV5 P-k alone

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Figure taken from Randall and Bermingham, 1996.

between polymeric NP and P could be identified. Two approaches were taken to identify the SV5 P-e epitope. These examined the ability of SV5 P-e to recognise either a series of C-terminal peptides immobilised on a nitrocellulose filter or a series of C-terminal deletion mutants.

3.2.1 mAb epitope mapping using SPOTS membrane

To identify the epitopes of the P unique mAbs, including SV5 P-e, a series of peptide spots, each of 13 amino acids, and with 8 amino acid overlaps, covering the whole of the P unique C-terminal region (amino acids 163-393), were generated on a nitrocellulose filter (Genosys), (See Materials and Methods Chapter 2, Section 5.8). This 'SPOTS' membrane was then incubated with each of the P unique mAbs individually to define the individual binding sites within the C-terminal region of P. First, the 'SPOTS' membrane was blocked O/N at 4 °C in a casein based blocking buffer supplied by the manufacturer. This prevented non-specific binding of the test mAb with the membrane (c.f. western blot). The mAb of interest (approximately 0.1 μg/ml in blocking buffer) was then incubated with the membrane for 30 mins at room temperature with shaking. Unbound mAb was then removed by washing the filter extensilvely in PBS containing 0.05% Tween 20 (PBS-T). Any mAb bound to the peptide spots was reacted with HRP conjugated sheep anti-mouse IgG. This secondary antibody was detected in an ECL reaction as with a western blot. The membrane was regenerated after incubation with one mAb to allow the mapping of each mAb binding site(s) individually (See M+M for details on membrane regeneration).
Fig.46 shows an outline of the SPOTS membrane when probed with sheep anti-mouse HRP alone and demonstrates that there was no non-specific interaction between the secondary antibody (sheep anti-mouse HRP) and the bound peptides. It also shows the relative positions of the peptide dots on the membrane. SV5 P-d bound to peptide spot number 4 which corresponded to amino acids 178-190 and SV5 P-a bound to spot number 6 which corresponded to amino acids 188-200, both in the C-terminal region of the P protein, as expected. Although successful for 2 of the 8 mAbs tested, the binding

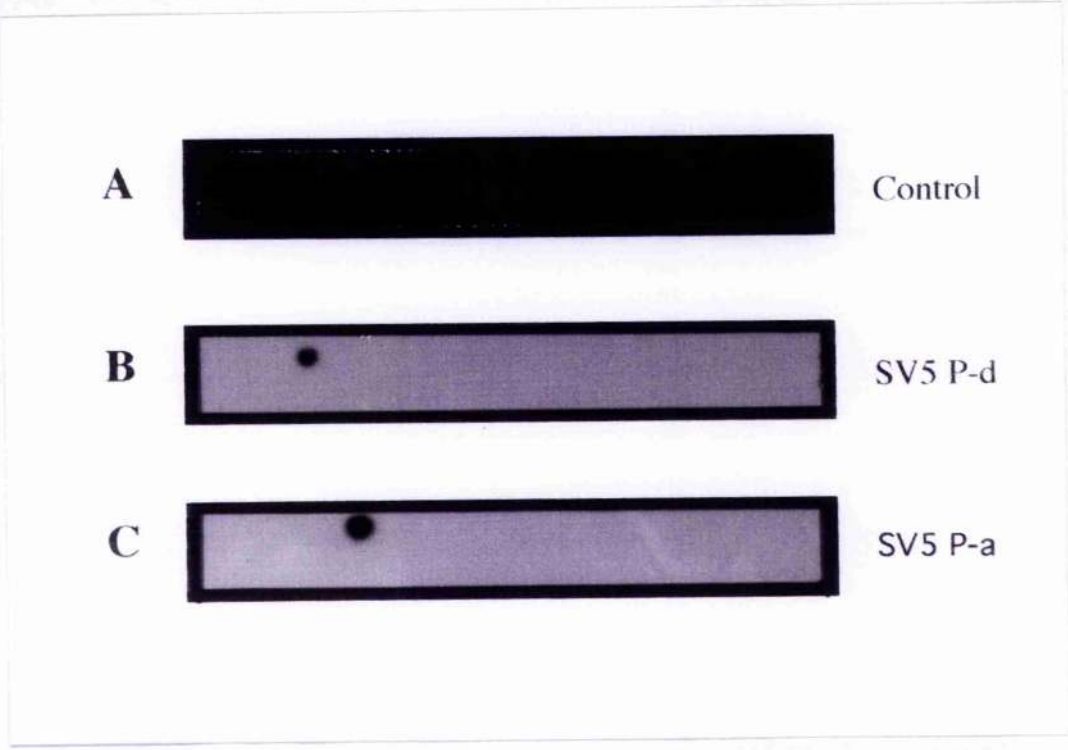

Fig.46:Mapping of mAb binding sites in C-terminus of P

SPOTS membrane containing overlapping peptides from the unique C-terminal region of P was probed with a panel of mAbs (individually) which had been raised against this region in order to map their epitopes. Panel A shows the membrane probed with anti-mouse Ig conjugated to HRP and detected in an ECL reaction. The positions of the peptide spots can be seen as clear patches on the darker background and demonstrated that there was no cross-reaction between the anti-mouse Ig conjugated HRP and the bound peptides. Only 2 of the 8 mAbs tested bound to the membrane. Panel B shows the binding site of SV5 P-d corresponded to spot 4 (amino acids 178-190 SDGWEMKSRSLSG) and Panel C shows the binding site of SV5 P-a corresponded to spot 6 (amino acids 188-200 LSGAIHPVLQSPL). Once bound to the membrane, the mAbs were detected with anti-mouse Ig conjugated HRP and detected in an ECL reaction as with western blots.

sites of the other mAbs, including SV5 P-e, could not be determined using the SPOTS membrane.

3.2.2 Cloning of C-terminally deleted mutants

An alternative strategy was employed to elucidate the SV5 P-e binding site using a series of C-terminal deletion mutants. Three unique restriction enzyme sites found in the C-terminal region of P were used to create truncated ORFs which were then cloned into pTM1 for protein expression from the VacT7 system.

The cloning of the P deletion mutants is outlined in Fig.47. The P ORF was initially isolated from pGEM-P by digestion with EcoR1 and Sal1 restriction enzymes. Once purified, the P ORF was digested again with either Ear1, Sma1 or Sca1 restriction enzymes and subsequently digested with Nco1. The fragments of interest were gel purified and ligated to pTM1 which had been digested with Nco1 and Stu1 restriction enzymes. The resultant plasmids were called pTMP-Ear1, pTMP-Sma1 and pTMP-Sca1 respectively. (Note that Ear1, Sma1, Sca1 and Stu1 are blunt cutters and blunt ends of the insert were ligated to the vector.)

3.2.3 Modification to the original capture assay

The C-terminal deletion mutants had been constructed for expression in the VacT7 system and subsequent examination by capture assay. Before these assays were performed, a modification to the capture assay protocol was made and used in all subsequent capture assays.

The original capture assay methodology described formaldehyde cross-linking of the *S.aureus* to the microtitre plate followed by addition of the mAb of interest. When the proteins in each well were analysed by western blot, the heavy and the light chains from the immobilising antibody were visualised, and in some cases, obstructed the band of interest from being seen clearly. (e.g. Fig. 44 Panel d, Lanes 1, 2 and 3, where

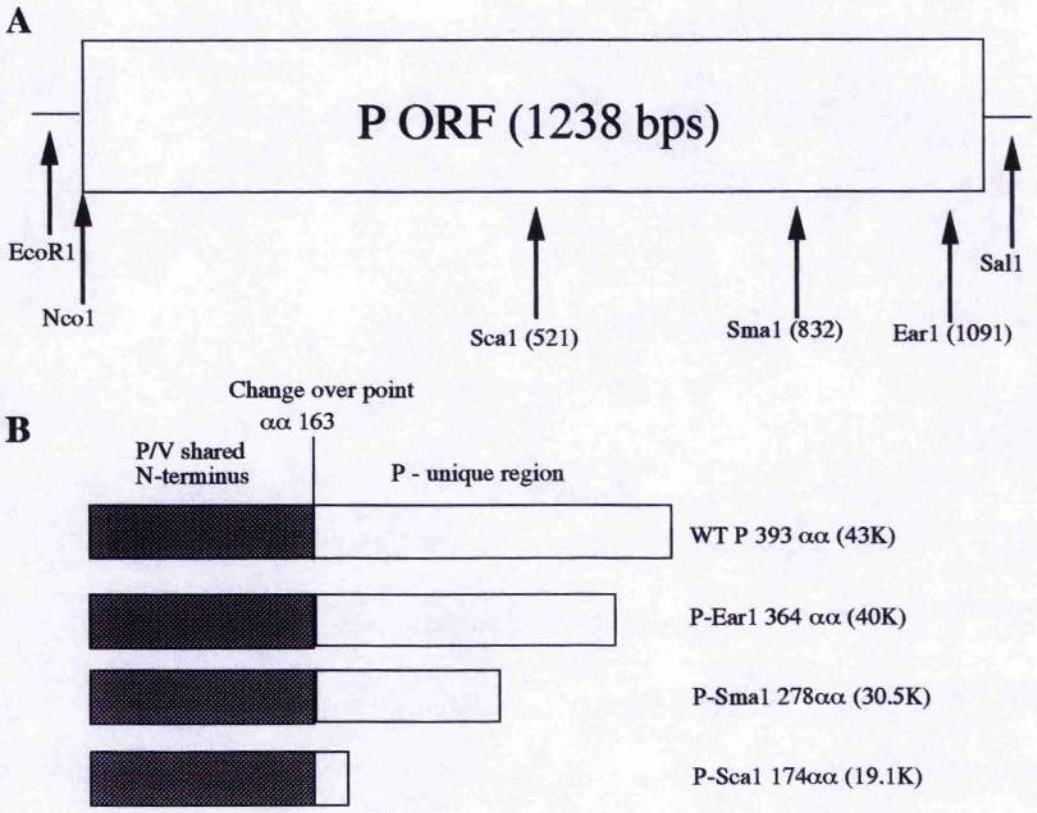

Fig.47: Construction of P deletion mutants

P ORF was excised from pGEM-P by digestion with EcoR1 and Sal1 restriction enzymes. Once purified, the ORF was further digested with Nco1 and either Sca1, Sma1 or Ear1 and purified again for ligating to pTM1 which had been digested with Nco1 and Stu1. The resultant plasmids were called pTMP-Sca1, pTMP-Sma1 and pTMP-Ear1. Panel A shows the positions of the restriction sites within the P ORF and Panel B show the relative sizes of the resulting mutant P proteins. The P/V shareN-terminus is shown as a shaded box and the P-unique region is also indicated.

migration of V is masked by the detection of light chain from the immobilising antibody, SV5 P-k.) To circumvent this problem, formaldehyde was used to cross-link the mAb of interest to the *S.aureus* on the plate. It was found from a time course experiment that 3 hours was the minimum incubation time which gave effective cross-linking of the mAb to *S.aureus* (Data not shown). Once the mAb was cross-linked to *S.aureus*, the capture assay proceeded as described previously. It should be noted that the cross linking of the mAb to *S.aureus* merely cut down on background signal from the immobilising antibody but did not completely erase it.

3.2.4 Expression of P deletion mutants

Once cloned, pTM-P, pTMP-Ear1, pTMP-Sma1 and pTMP-Sca1 were transfected into VacT7 infected 293 cells for protein expression. The cell extracts were prepared as described in Materials and Methods, Chapter 2, Section 5.14.4 and 5.24.5. The migration of the C-terminally deleted proteins and their ability to be recognised by SV5 P-k, was examined by capture assay. A StaphA plate, containing SV5 P-k, was prepared as described previously and the VacT7 generated protein extracts were diluted 1/100 in PBS before addition to the plate. The extracts were incubated and harvested as described previously with the resultant western blot shown in Fig.48. All of the P proteins were recognised by the N-terminal mAb SV5 P-k. Full-length P protein was expressed from pTM-P and is referred to as wild-type P (WT-P) in Panel A, Lane 3. The deletion mutants, pTMP-Ear1, pTMP-Sma1 and pTMP-Sca1 are shown in Lanes 3 of Panels B, C and D respectively. The relative mobilities of all the recombinant P proteins were compared to the authentic viral protein from an SV5 infected cell extract (Lane labelled ICE in Panels A, B, C and D.).

3.2.4 Mapping of SV5 P-e binding site with deletion mutants

The ability of the C-terminal deletion mutants to be recognised by SV5 P-e was then tested, with the aim of identifying the location of the mAb binding site. This method

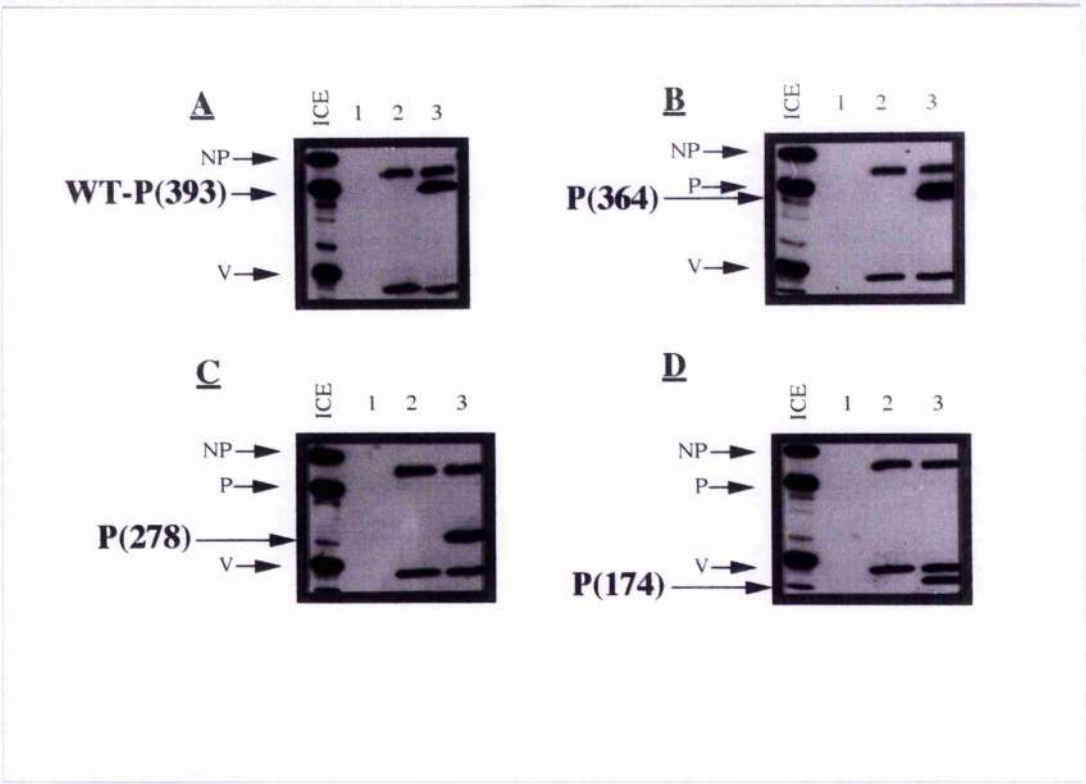

Fig.48: VacT7 expression of P deletion mutants in capture assay

Western blot demonstrating the ability of VacT7 driven expression of P deletion mutants from pTM vectors to bind SV5 P-k in capture assay. The mAb SV5 P-k was added to *S. aureus* coated 96 well microtitre plates. (Lanes 2 and 3 Panels A, B, C and D respectively) Lane 1 in each panel represents *S. aureus* alone and Lane 2 represents mAb SV5 P-k bound to *S. aureus* . Cell extracts from 293 cells infected with VacT7 and transfected with pTM-P (and derived P deletion mutants) were prepared as described in the text. These cell extracts (or PBS, Lane 2 in each panel) were added to the capture plates (Lane 3 in each panel) and incubated for 3 hours at 4 °C with rocking. Unbound material was removed by extensively washing with PBS and polypeptides present in the wells, and those from an SV5 infected cell extract (ICE) were electrophoresed through 10% SDS-PAGE and western blotted. The P protein was detected with mAb SV5-P-k and NP protein was detected with the mAb SV5 NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig.

The 393 amino acid wild-type or full length SV5 P protein expressed from a VacT7 driven pTM-P clone is indicated by **WT-P(393)**. P proteins from the deletion mutants expressed in the same way are indicated by capital P (**P**) with protein length (in amino acids) given in brackets.

was tested by first using the mAb, SV5 P-a where the binding site had already been determined using the SPOTS membrane. This mAb was used to coat a StaphA plate whereupon extracts containing the full-length or C-terminally truncated P proteins were added. Bound polypeptides were harvested as described previously and the resultant western blot is shown in Fig.49, Panel C. Full length P (393 amino acids) was bound by SV5 P-a (Lane 3) as were P-EarI (364 amino acids) and P-SmaI (278 amino acids) in lanes 9 and 7 respectively. However, P-ScaI (174 amino acids) was not bound by SV5 P-a (Lane 5) indicating that the binding of the mAb lay between amino acids 174 and 278. This is consistant with the SPOTS analysis where the binding site had been shown to be amino acids 188-200 in the C-terminus of P.

Since this method had been shown to work, the experiment was repeated with SV5 P-e bound to a StaphA plate. The extracts were incubated as described previously and the resultant western blot is shown in Fig.49, Panel B. Full length P was bound by SV5 P-e (Lane 3) but none of the mutants were recognised by the mAb (Lanes 5, 7 and 9). This demonstrated that deleting the extreme C-terminal 29 amino acids, abolished the ability of the P protein to be recognised by SV5 P-e, indicative of the binding site being in this region. Therefore the putative binding site on P for interactions with polymeric NP lies, at least in part, within the extreme C-terminal 29 amino acids.

3.3 NP interactions with bacterially expressed P and V

For future examination of structure / function relationships in both P and V proteins, it was necessary to generate large amounts of protein for X-ray crystallographic studies. Neither the inducible cell lines nor the VacT7 system could generate sufficient amounts of protein for this purpose and so bacterial expression of both P and V proteins was investigated. The pET (plasmid for Expression by T7 polymerase) system was chosen since it is one of the most efficient ways of expressing high levels of protein in *E.coli* (See Materials and Methods, Chapter 2, Section 5.14.4 for details on the pET system) and would facilitate the generation of both clones and protein for future mutational analysis. Before any X-ray crystallographic studies were undertaken, some indication of

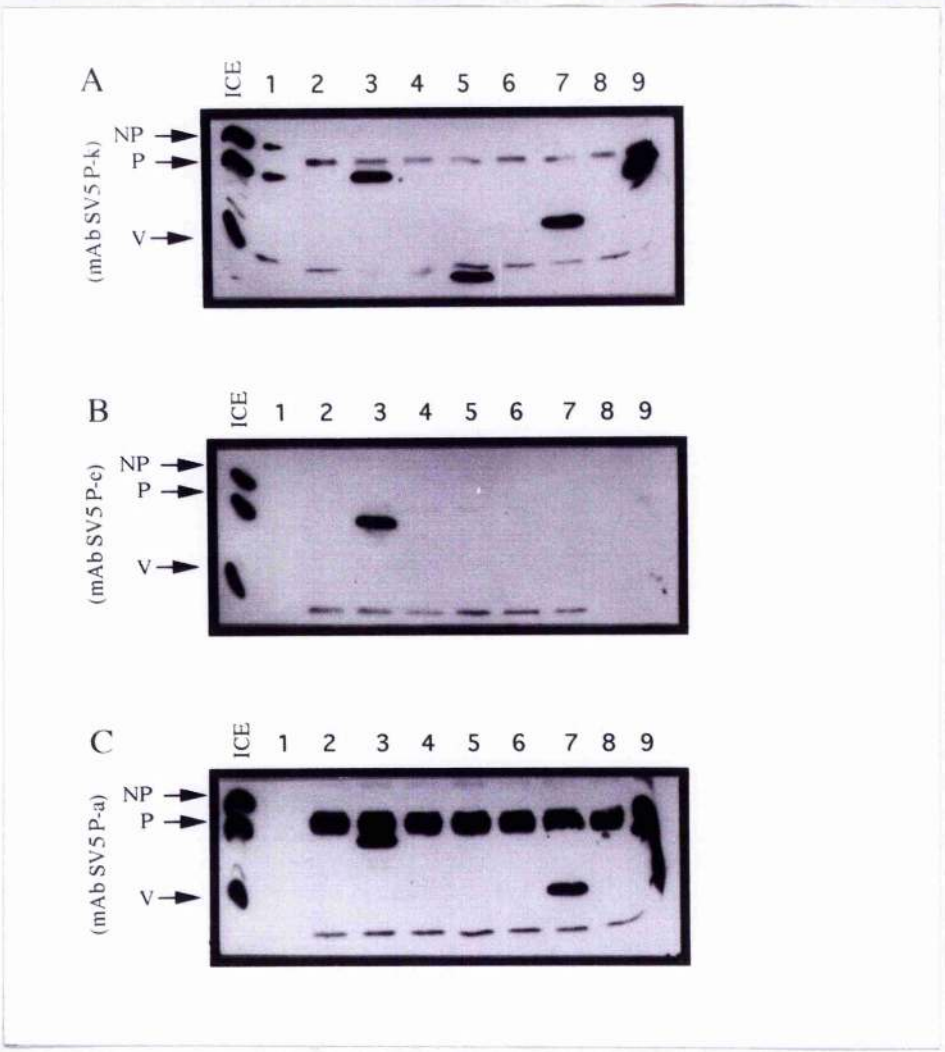

Fig.49 : Mapping of SV5 P-e binding site by deletion mutant analysis.

S.aureus microtitre plate was prepared and the mAb SV5 P-k was added to wells 2-9 of Panel A, mAb SV5 P-e was added to wells 2-9 of Panel B and mAb SV5 P-a was added to wells 2-9 on Panel C. Extracts from VacT7 generated pTM-P and P deletion proteins were prepared as described in the text. An extract containing full-length P (393 amino acids) was added to Lane 3 in Panels A, B and C respectively. P-ScaI (174 amino acids) was added to Lane 5 in Panels A, B and C respectively. P-SmaI (278 amino acids) was added to Lane 7 of Panels A, B and C respectively. P-EarI (364 amino acids) was added to Lane 9 of Panels A, B and C respectively. (PBS was added to wells 1, 2, 4, 6 and 8 of Panels A, B and C respectively. Unbound material was removed by washing extensively in PBS whereupon the residual poypeptides (including those of an infected cell extract) were seperated by electrophoresis through a 10% SDS-polyacrylamide slab gel and analysed by western blot as described previously.

Note that Lane 1 of Panel A contained *S.aureus* alone and therefore the bands seen in this lane are 'spill-over' from the infected cell extract (ICE) control lane.

the ability of the bacterially expressed protein to behave as native protein was necessary. The ability of the bacterially expressed proteins to be recognised by both N-terminal and C-terminal mAbs would suggest that the proteins had folded corectly. This would be further strengthened if the proteins could form complexes with NP similar to those made by cell line expressed P and V proteins.

3.3.1 Construction and Expression of pET-P and pET-V

Both pET-P and pET-V were constructed by Mr. B.L. Precious and used by me in the experiments described below. The P and V ORFs were directly subcloned from pGEM-P and pGEM-V into the bacterial expression vector, pET11c, for IPTG inducible expression in *Escherichia coli*. Bacterial cells containing the pET-P or pET-V plasmids were grown and induced with 1 mM IPTG as described in Materials and Methods, Chapter 2, Sections 5.14.4 and 5.14.5. Bacterial cell extracts were prepared by treating the cells with lysozyme and then diluting the lysates in IP buffer (without SDS). Lysates were sonicated and cell debris was pelleted, whereupon the supernatant was decanted into a fresh tube. The resulting supernatant was diluted 1/1000 in PBS for addition to StaphA plates.

3.3.2 Bacterially expressed P and V bind NP

Bacterially expressed P and V were initially assayed for their ability to be recognised by the N-terminal mAb, SV5 P-k, on a StaphA plate. A StaphA plate was therefore prepared and coated with SV5 P-k as described previously. 100 µl of bacterially expressed P or V protein, or 100 µl of cell expressed P or V protein was added to the appropriate wells and incubated for 2 hours at 4 °C with rocking. The plate was extensively washed with PBS to remove unbound material. The bound polypeptides were harvested as described previously for electrophoresis and western blot analysis. Fig.50 demonstrated that both bacterially expressed P (Panel B, Lane 3) and V (Panel

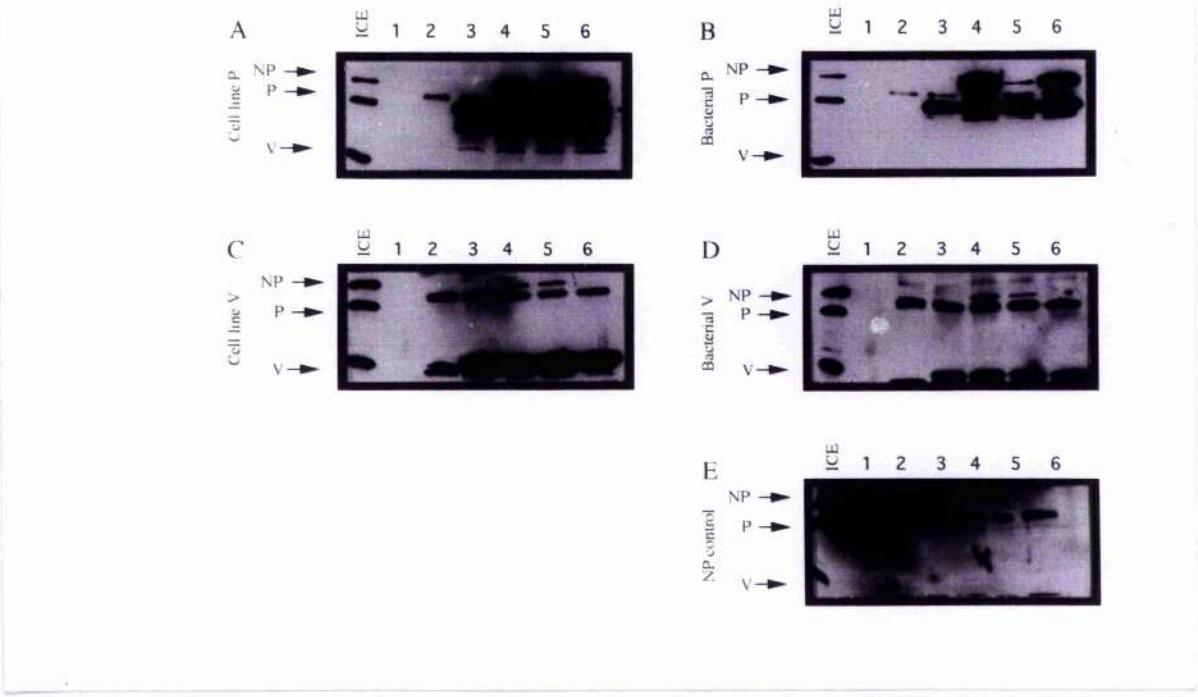

Fig.50: Bacterially expressed P and V can bind NP.

Western blot comparing bacterially expressed P and V proteins with BalbC cell line extracts containing P and V proteins for their ability to bind NP by capture assay. The mAb SV5-P-k was bound to *S. aureus* coated wells of a 96 well microtitre plate. Cell extracts from BalbC cell lines expressing P (Panel A, Lanes 3-6), or V (Panel C, Lanes 3-6) were added to the plate. Bacterial lysates were prepared as described in the text and bacterially expressed P (Panel B, Lanes 3-6) or V (Panel D, Lanes 3-6) was added to the plate and incubated at 4 °C for at least 2 hours on a rocker. After washing extensively with PBS, low speed supernatant, high speed supernatant and high speed pellet extracts of cells expressing NP protein were added to lanes 4, 5 and 6 respectively of Panels A, B, C, D and E and incubated for 3 hours on a rocker at 4 °C . Lanes 1, 2 and 3 in panels A, B, C and D contained *S. aureus* alone, *S.aureus* plus mAb SV5 P-k, and *S. aureus* plus mAb SV5 P-k plus appropriate P or V extract respectively. Panel E represents a negative control for non-specific interaction between the NP extracts and mAb SV5 P-k bound to the plate. Lanes 1 and 2 contained *S. aureus*, *S. aureus* plus mAb SV5 P-k respectively. Following removal of the unbound material by washing extensively with PBS, the polypeptides present in the wells, and those from an SV5 infected cell extract (ICE),were electrophoresed through a 10% SDS-PAGE and western blotted. The P and V proteins were detected with mAb SV5-P-k and NP protein was detected with the mAb SV5 NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig.

D, Lane 3) were recognised to the same degree as their cell line counterparts (Lanes 3 of Panels A and C, respectively). This suggested that at least the N-terminal region of the bacterially expressed proteins had folded sufficiently for recognition by the N-terminal mAb, SV5 P-k.

The bacterially expressed proteins were subsequently assayed for their ability to form complexes with NP, in a repeat of the experiment in 3.1.4. A StaphA plate containing SV5 P-k was prepared as described previously. 100 µl of the bacterially expressed P or V proteins was added to the appropriate wells (Cell line expressed P and V were included as positive controls).The plate was incubated for 1 hour with shaking at 4 °C, and the unbound material was removed by washing extensively with PBS. 100 µl of LSS, HSS and HSP extract from NP expressing cells were added to the plate and incubated for a further hour at 4 °C, with shaking. Again the unbound material was removed by washing in PBS and the bound polypeptides were harvested as decribed previously, for electrophoresis and western blot analysis.

The bacterially expressed proteins displayed binding profiles identical to those of the cell line expressed proteins. Bacterially expressed P bound both soluble and polymeric NP (Fig.50, Panel A, Lanes 4, 5 and 6), while bacterially expressed V bound only the soluble form of NP (Panel C, Lanes 4, 5 and 6), thus mirroring the result obtained with the cell line expressed P and V proteins (respectively Panels B and D, Lanes 4, 5 and 6, and section 3.1.4). Furthermore, the P and V proteins had been shown to be phosphorylated when expressed in their respective BalbC cell lines (Precious *et al*, 1995). When expressed in bacteria, however, no post-translational modification associated with the mammalian expression system would have taken place. Therefore, the results above, demonstrate that the phosphorylation states of either P or V proteins, did not influence their ability to bind NP.

3.3.3 SV5 P-e blocks bacterially expressed P binding to polymeric NP

To further strengthen the findings that bacterially expressed P behaved in an identical fashion to its cell line expressed counterpart, the ability of the protein to be recognised

by the C-terminal mAb, SV5 P-e, was tested. A StaphA plate containing either SV5 P-e or SV5 P-k (as a positive control for P binding), was prepared. Bacterially expressed P was added to the plate and the bound polypeptides were assayed by western blot. Fig.51 confirms that bacterially expressed P is recognised by the SV5 P-k (Panel A, Lane 3) but also demonstrates that SV5 P-e recognises the bacterially expressed protein (Panel B, Lane 3).

The ability of the C-terminally captured P protein to bind NP was then assayed. For this, LSS, HSS and HSP extracts of NP expressing cells were added to a StaphA plate containing bacterially expressed P protein captured by SV5 P-k or SV5 P-e. NP-P interactions were analysed by western blot as described previously. Once again, the bacterially expressed P captured by SV5 P-k bound both soluble and polymeric forms of NP (Fig.51, Panel A, Lanes 4, 5 and 6) and SV5 P-e blocked the interaction between bacterially expressed P and polymeric NP (Panel B, Lanes 4, 5 and 6). This result once more mirrored that seen with P expressed im mammalian cells (3.1.5).

Both P and V proteins expressed in *E.coli* had NP binding profiles in capture assays indistinguishable from their mammalian cell line counterparts. They were recognised by both N-terminal and C-terminal mAbs, suggesting that, even though they were without the normal post-translational modifications, they had not taken up a totally aberrant structure. Therefore, the bacterially expressed P and V proteins were deemed suitable for X-ray crystallography and future structure/function analysis.

3.4 Summary and Conclusions from Capture Assays

Fractionation of extracts from the NP expressing cell line demonstrated that there were two separable populations of NP expressed in the cells; one soluble and the other polymeric. Both P and V proteins bind soluble NP suggesting that the site of interaction between P or V and soluble NP lies in the N-terminus of the protein as P and V are amino-coterminal. For SeV, P protein has been shown to act as a chaperone for NP by forming a soluble NP:P complex. This prevents NP from self-aggregating and also acts as the encapsidation substrate during both genomic and anti-genomic RNA synthesis

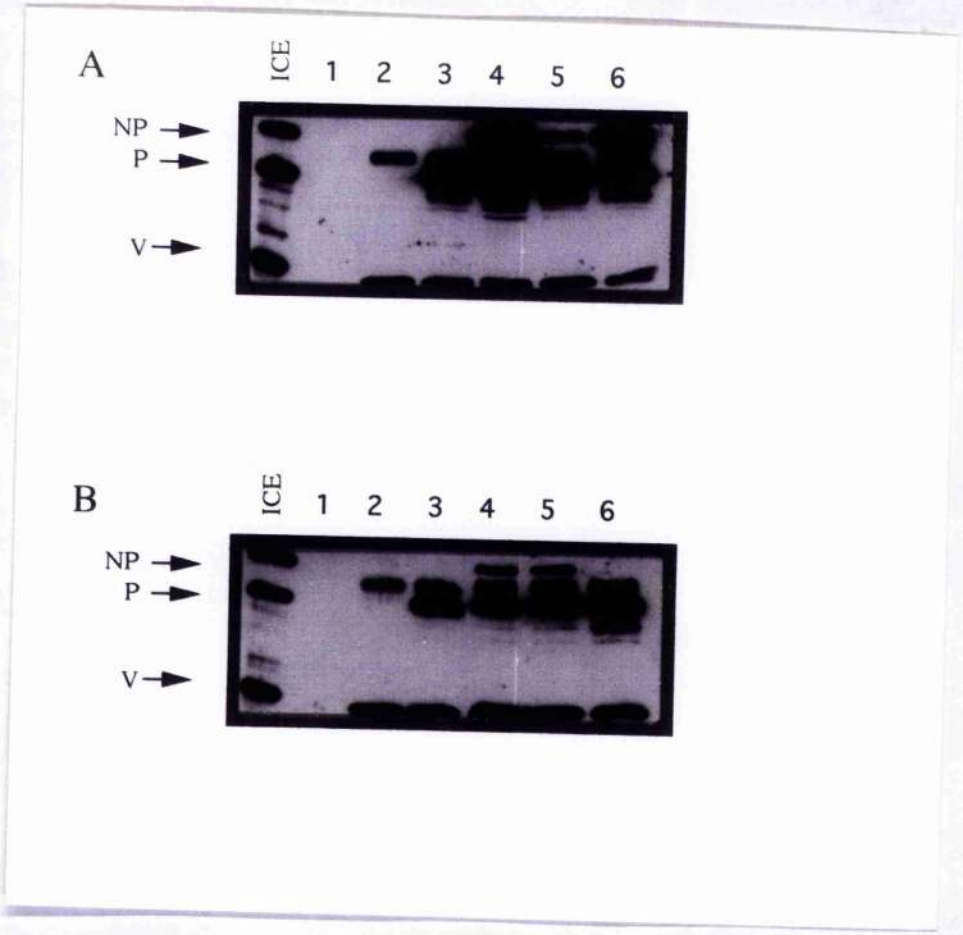

Fig.51:Binding of bacterially expressed P to polymeric NP can be blocked by mAb SV5 P-e.

Western blot demonstrating the ability of mAb SV5 P-e to prevent the binding of bacterially expressed P to the polymeric form of NP. The mAbs SV5 P-k and SV5 P-e were added to *S. aureus* coated 96 well microtitre plates. (Lanes 2-6 Panels A and B respectively) A bacterial cell extract was prepared as described in the text and added to wells 3-6 for 3 hours at 4 °C on a rocker. Unbound material was removed by washing extensively in PBS before addition of low speed supernatant, high speed supernatant and high speed pellet extracts of NP cell line to lanes 4, 5 and 6 respectively. Lanes 1 and 2 in Panels A and B contained *S. aureus* alone and *S. aureus* plus mAb respectively. Following reomoval of the unbound material by washing extensively with PBS, the polypeptides present in the wells, and those from an SV5 infected cell extract (ICE), were electrophoresed through a 10% SDS-PAGE and western blotted. The P protein was detected with mAb SV5-P-k and NP protein was detected with the mAb SV5 NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig.

(i.e. virus replication).The data presented in this section support this finding by demonstrating that SV5 P can interact with soluble NP. Furthermore, V can also interact with soluble NP, suggesting that V could fulfil a similar chaperone role in SV5, to that seen with P for SeV. This possibility is discussed further in Chapter 4.

P also binds polymeric NP, suggesting that this site of interaction lies within the unique C-terminus of P. This site was subsequently predicted to reside, at least in part, within the extreme C-terminal 29 amino acids. These results also support findings for SeV P protein, which has been shown to interact with the viral nucleocapsid (assembled NP). This interaction is thought to occur when P is in the P-L polymerase complex and thereby anchors the polymerase to the template for either transcription or replication. This is again discussed in more detail in Chapter 4.

Therefore, the protein : protein interactions demonstrated in this section give some insight into the roles played by these proteins within the replication complex.

Chapter 4 : Discussion

As described in Chapter 1, a number of methods are now available to study the transcription / replication mechanisms in negative strand RNA viruses. Most, if not all, involve the use of a reverse genetics system of one kind or another. Unfortunately, the reverse genetics approaches described here proved unsuccessful for the investigation of SV5 biology. However, the results presented in this thesis provide evidence for protein : protein interactions within the replication complex and possible roles for these interactions during virus replication are examined.

This discussion is therefore divided into 4 sections. The first deals with the problems associated with our reverse genetics experiments and discusses approaches taken in more successful systems. The second discusses viral protein : protein interactions within the replication complex. Thirdly, a model for SV5 replication is proposed, with respect to the protein : protein interactions presented in Section 2. Finally, section 4 outlines future applications of genetically engineered negative-strand RNA viruses.

1 Reverse Genetics of Negative Strand RNA viruses

1.1 Problems associated with our SV5 rescue system

During the course of this study, the generation of a reverse genetics system to study SV5 biology proved unsuccessful. Initial experiments utilized a le-CAT-tr genomic (negative-sense) RNA analogue which was transcribed intracellularly from a transfected cDNA clone under T7 promoter control. Synthesis of the synthetic analogue was driven by T7 DNA-dependent RNA polymerase, supplied by a recombinant vaccinia virus expressing the polymerase (Chapter 3, Section 1.2). However, it was found that the recombinant vaccinia virus (VacT7) could generate CAT mRNA directly from the cDNA template. Although the reason(s) for this were never determined, similar results were found in other laboratories (Dr. C. Ward, Northwestern University; Dr. E. Dunn, Glasgow University; personal communication), and can perhaps be attributed to the promiscuity of the vaccinia virus DNA-dependent RNA polymerase. The polymerase is known to have a consensus core recognition region with no apparent sequence requirements upstream of the core or downstream of the initiation site (Reviewed in Moss, 1996). It is possible that a sequence similar to this core recognition region had been serendipitously encoded within the le-CAT-tr cDNA clone thus allowing CAT mRNA transcription directly from the cDNA template.

Alternative methods of generating (and delivering) synthetic CAT transcripts were also investigated but irrespective of the method employed for the generation of an SV5 genome analogue, no CAT activity was detected, suggesting that the input CAT RNA had not been transcribed.

It was possible to mimic the molar ratios of the encapsidation proteins (NP, P, V and L) seen in an SV5 infection by co-transfecting their respective cDNA clones. Ratios of input plasmid DNA were adjusted until target levels of protein expression, driven by VacT7, were achieved. This strategy was used to supply viral proteins *in trans* for the encapsidation and transcription of the le-CAT-tr RNA transcript, but was unsuccessful.

Possibly the synthetic nucleocapsid structure was not recognised by the viral polymerase, regardless of whether it was supplied by a cDNA clone or by infectious virus. However, RNAse protection assays provide some evidence to suggest that the CAT transcript may not have been encapsidated (Dr. C. Ward, personal communication) leading to the possibility that the necessary encapsidation substrate had not been formed. The protein : protein interactions directing encapsidation,and replication were therefore examined using inducible cell lines expressing SV5 proteins. The distribution of the encapsidation proteins (NP, P and V) when expressed alone or in combination was examined by IF and extracts from these cell lines were subsequently used in capture assays to study the interactions in more detail, as discussed in section 2.

1.1 Rescue of infectious recombinant viruses

A successful reverse genetics system has now been developed for SV5 using a cDNA clone of the complete genome, which was constructed such that an anti-genome (positive sense) RNA could be transcribed by T7 RNA polymerase. This plasmid was co-transfected with cDNA clones encoding the viral replication proteins (NP, P and L) resulting in the isolation of infectious recombinant virus (He *et al*, 1997).

The differences in sense between the respective SV5 RNA analogues (positive sense full-length genome analogue versus negative sense le-CAT-tr mini-genome), may have contributed to their ability to be rescued, but it remains unclear why a positive sense anti-genome analogue would be intrinsically easier to encapsidate that its negative sense counterpart. Recent reports on the recovery of recombinant viruses such as rabies virus (Schnell *et al*, 1994), VSV(Lawson *et al*, 1995), MeV (Radecke *et al*, 1995) and SeV (Garcin *et al*, 1996) do suggest that the use of anti-genomic transcripts may tip the balance in favour of successful rescue.

However, a series of experiments published in a recent report demonstrated that intracellular expression of a full-length transcript generated infectious SeV regardless of whether the input transcript was positive or negative sense (Kato *et al*, 1996). Success appears to have come by fine tuning the amounts of NP, P and L proteins used in the system, constructing plasmids which gave rise to transcripts with 3' and 5' ends

identical to those of wild-type RNA, and by using vaccinia virus inhibitors (rifampicin and cytosine arabinoside) to reduce the cytotoxicity of the vaccinia virus.

Once the system had been optimized, these authors found that infectious SeV could be recovered by transfecting naked RNA. This supports earlier reports where naked mini-genome RNAs were amplified and expressed in cells infected with RSV (Collins *et al*, 1991), hPIV3 (De and Banerjee, 1993; Dimock and Collins, 1993) and SeV (Park *et al*, 1991).

It is interesting to note that the successful SV5 rescue system mentioned above, has yet to support amplification and expression from a le-CAT-tr mini-genome construct, suggesting that rescue of the CAT mRNA may be more difficult than rescue of a full-length infectious clone. Perhaps this can be explained by considering the efficiency of rescue of a recombinant virus versus CAT mRNA. In a similar rescue system to that used for SV5, it was calculated that one per 10^7 cells contained recombinant rabies virus (Schnell *et al*, 1994) and a similar success rate might be predicted for SV5. Mini-replicons (e.g. le-CAT-tr) do not encode the viral proteins necessary for autonomous replication and therefore need a constant supply of viral proteins for their propagation. Therefore, the mini-genome system would be expected to be much less efficient due to the lack of autonomous transcription / replication and consequently would be more difficult to detect.

2 Protein:Protein Interactions within the Replication Complex

2.1 Protein : Protein interactions seen by IF

Immunofluorescence data demonstrated that when NP was expressed alone in an inducible cell line, it formed cytoplasmic aggregates, visualised as punctate and granular foci (Chapter 3, Section 2.2). This is consistent with a number of other studies on paramyxoviruses which have reported that NP forms homopolymers when expressed alone (Buchholz *et al*, 1993; Horikami *et al*, 1996; Spehner *et al*, 1997). During this self-assembly process, cellular RNA can be non-specifically encapsidated (B. Precious, unpublished observation) into an RNase resistant form (Buchholz *et al*, 1993; Horikami *et al*, 1996; Spehner *et al*, 1997). Moreover when co-expressed with P, NP formed large cytoplasmic aggregates similar to the inclusion bodies seen at late times in SV5 infection (Chapter 3, Section 2.2). In a recent report with MeV, NP and P co-expression led to the formation of similar large cytoplasmic aggregates (Spehner *et al*, 1997), supporting our results obtained with SV5.

These findings seemed to contradict a report in the SeV system where P had been proposed to act as a chaperone in keeping NP soluble prior to encapsidation (Curran *et al*, 1995). The recent study with MeV (Spehner *et al*, 1997), showed that although co-expression of NP and P proteins ultimately led to the formation of large cytoplasmic aggregates, P did form soluble complexes with NP. These soluble NP-P complexes retained the ability to self-assemble into nucleocapsid-like structures, but to a lesser extent than when NP was expressed alone. Therefore by analogy to MeV, during ongoing viral replication in both SV5 and SeV, soluble NP-P complexes may be formed which deliver soluble NP to the elongating RNA chain during encapsidation. However, in the absence of viral replication, the NP-P complexes may self-assemble into the large cytoplasmic aggregates which, for SV5, were visualised by immunofluorescence as inclusion bodies.

When co-expressed with V, NP expression was found to give diffuse cytoplasmic and nuclear fluorescence, suggesting that V prevented the formation of NP aggregates by forming soluble NP-V complexes (Chapter 3, Section 2.2). As yet, there is no evidence to suggest that NP-V complexes can self assemble in a similar manner to that seen with NP-P and possible reasons for this are discussed below.

2.2 Protein : Protein interactions by capture assay

Data from the capture assays demonstrated direct viral protein : protein interactions within the replication complex and the results are summarised in Fig. 52. Firstly, centrifugation of an extract from NP expressing cells demonstrated that NP was found in both soluble and polymeric forms (Fig. 52, Panel A). For MeV NP, a phosphorylation event seems to trigger a conformational change in NP, thus allowing assembly into nucleocapsid structures (Gombart *et al*, 1993). Perhaps a similar phosphorylation event and conformational change is required by SV5 NP prior to assembly, which has yet to be demonstrated experimentally.

Both P (Fig 52 Panel Cii, Panel Dii and Panel Diii) and V (Fig.52 Panel Bii) proteins were shown to bind the soluble form of NP by capture assay, suggesting each protein could act as a chaperone (similar to MeV or SeV P proteins) in keeping NP soluble prior to encapsidation. The P and V proteins are amino co-terminal and therefore the site of interaction between P/V and soluble NP was predicted to be in this shared N-terminal domain. It is possible that binding of V to soluble NP blocks the proposed phosphorylation event (which may be necessary for NP assembly), resulting in a soluble NP:V complex which cannot self-assemble. Alternatively, the binding of V to NP may prevent NP self-assembly by blocking the NP:NP binding site. However, NP:P complexes seem to retain their ability to self-assemble, suggesting a difference in character between the NP:P and NP:V complexes.

Capture assays also demonstrated that V could not bind polymeric NP, therefore suggesting that V cannot bind nucleocapsids (Fig.52 Panel B iii). This contradicts a report by Paterson *et al*, (1995) which suggested that V formed part of the SV5 RNP structure. The study relied on immunogold labelling of purified viral nucleocapsids for

Fig. 52 NP:P and NP:V interactions

A schematic diagram summarising the results obtained by capture assay. Monomeric NP is depicted in Panel A(i) where the P/V common binding site and the second P binding site are clearly labelled. Polymeric NP is depicted in A(ii), where the P/V common binding site has been blocked by a conformational change in NP induced upon polymerisation or following a phosphorylation event.

V is shown alone in Panel B(i). The N-terminus of V interacts with soluble (monomeric) NP to form a soluble NP:V complex (B ii). However, V is unable to interact with polymeric NP (B iii).

Monomeric P is depicted in Panel C(i). Monomeric P may interact with soluble NP via the P/V shared N-terminus forming a soluble NP:P complex (Panel C ii). Monomeric P may also interact with polymeric NP via its extreme C-terminus (Panel C iii).

Trimerised P is depicted in Panel D(i).The number of monomeric NP molecules bound simultaneously by a P trimer, remains to be determined. Therefore, a P trimer may interact with a single NP molecule (Panel D ii) or perhaps with three NP molecules (Panel D iii). Panel D (iv) depicts the C-termini of the P trimer interacting with polymeric NP.

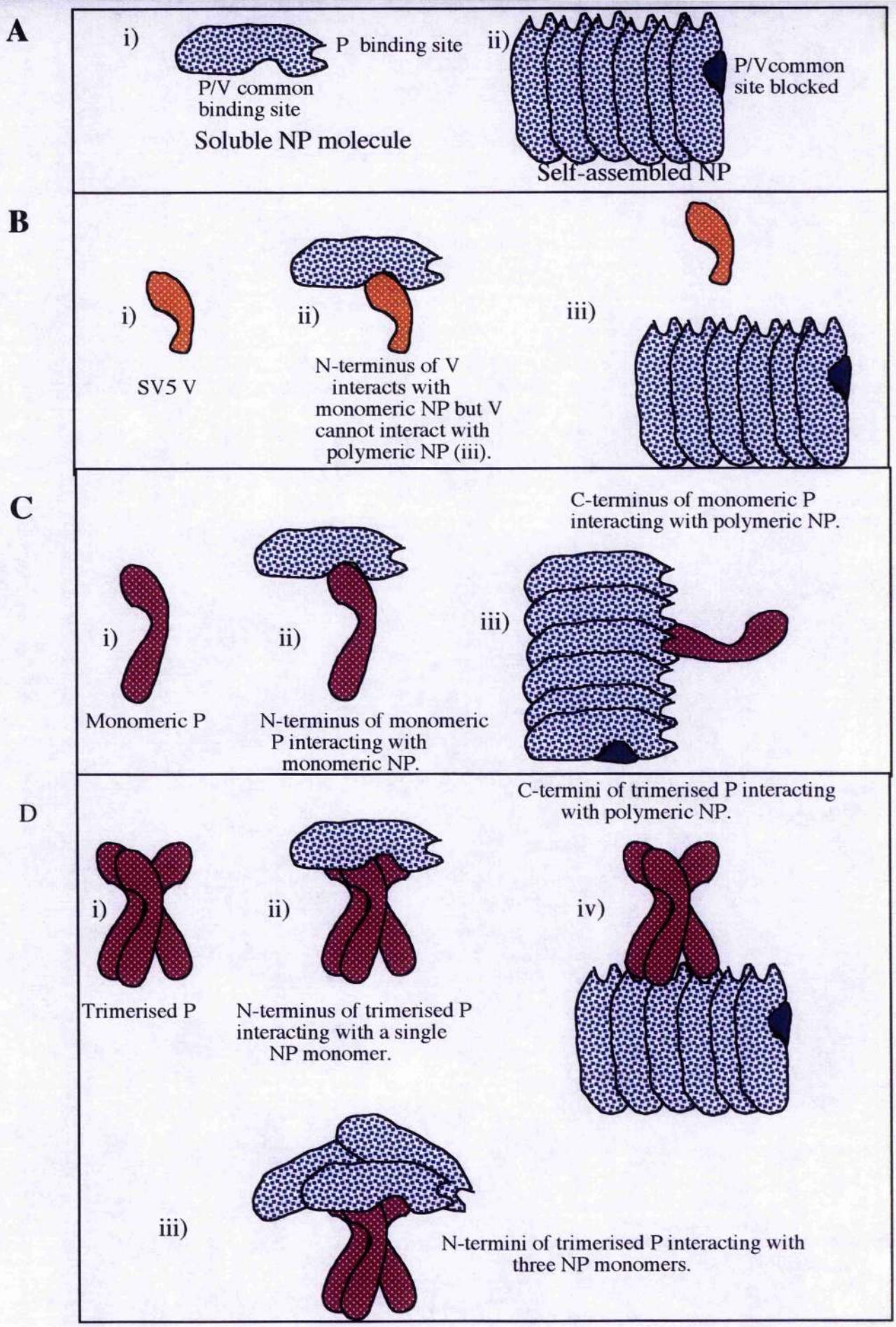

Fig.52 NP:P and NP:V interactions

examination by electron microscopy. The data presented, clearly showed V on the surface of the nucleocapsid. However, the paper was written with a caveat as the authors could not be sure if M (and its associated proteins) had been completely removed from the nucleocapsid preparations. Therefore it was not clear if V was binding the nucleocapsids or merely associated with the contaminating proteins. Alternatively, it is possible that V interacts directly with viral RNA. However, the capture assays presented in this thesis were performed many times with reproducible results, demonstrating that V cannot bind assembled NP. Therefore the alternate explanations offered by Paterson *et al* are favoured.

Since only P (and not V) could bind polymeric NP (Fig.52 Panels C iii and D iv), the P-unique C-terminus of the protein was predicted to contain the site of interaction with polymeric NP. Taken together, these data suggest there are two binding sites on P for NP; the soluble NP binding site, found in the N-terminus (common to both P and V proteins), and the polymeric NP binding site, found in the P-unique C-terminus. Furthermore, the results also suggest that there are two binding sites on NP for P; one on soluble NP which is recognised by both P and V proteins, and the other on polymeric NP, which is recognised by P only. When assembled, the P/V common binding site on NP must be either hidden or conformationally altered thus preventing recognition by V. If merely hidden, one would expect at least one site to be available for binding by V. No V interaction with polymeric NP has ever been detected so, although possible, it seems more likely that NP undergoes a conformational change upon polymerisation. Equally, this conformational change may have occurred prior to polymerisation following the proposed phosphorylation event mentioned previously. Either way, it seems likely that a conformational change in NP takes place which masks the common P/V binding site on polymeric NP (Fig.52 Panel A ii).

2.3 P interaction with polymeric NP

A mAb specific to the C-terminus of P, SV5 P-e, blocked the interaction of P with polymeric NP (Chapter 3, Section 3.2). This suggested that the site of interaction between P and polymeric NP contained, at least in part, the SV5 P-e epitope. By deletion mutant analysis of the C-terminus of the P protein, the extreme C-terminal 29

amino acids were predicted to contain the SV5 P-e epitope and therefore the site of interaction between P and polymeric NP.

However, one question remained unanswered. P is known to form a homotrimer and is thought to only bind the nucleocapsid as a trimer (Curran, 1996). Therefore, does SV5 P-e block P binding to polymeric NP by merely preventing P trimerisation? In SeV, the trimerisation domain was identified as amino acids 344-411, whereas in the *Rubulavirus*, hPIV2, this domain was recently identified as amino acids 211-248 (Nashio *et al*, 1997). The binding of SV5 P-e to P protein should not interfere with the analogous SV5 trimerisation domain. All of the P deletion mutants (except P-ScaI, 174 amino acids) would also be anticipated to form homotrimers since the predicted trimerisation domain in the mutants would be intact. Therefore, it is anticipated that SV5 P-e does not prevent P trimerisation but nonetheless blocks P binding to polymeric NP.

Since bacterially expressed P also binds polymeric NP and only trimerised P is thought to bind polymeric NP, then it could be suggested that bacterially expressed P must also form trimers (Chapter 3, Section 3.3). This suggests that P protein trimerises independently to its phosphorylation state, since bacterially expressed P trimers would have no post-translational modifications associated with the mammalian system.

It is not clear whether one molecule of P can bind both soluble and polymeric NP at the same time. Perhaps the binding of soluble NP prevents P from binding polymeric NP. If this was the case, then it is easier to visualise P acting as a chaperone for soluble NP.

2.4 The nuclear localisation signal

A nuclear localisation site has been identified in the V protein of hPIV2 and mapped to the extreme N-terminal amino acids 1-46 (Wantanabe *et al*, 1996). This study reported that when co-expressed with V, NP was transported to the nucleus in the absence of virus replication, supporting IF data presented in this thesis (Chapter 3, Section 2.2). What is curious, however, is that the domain in V identified as responsible for the nuclear targeting in hPIV2, (nuclear localisation signal), and so by analogy in SV5, is found in the P/V common domain, yet P is never found in the nucleus whether expressed alone or co-expressed with NP. This suggests that the nuclear localisation

signal in P is blocked, possibly due to a difference in conformation between the P and V proteins.

Trimerisation of P is thought to take place almost immediately after translation (Nashio *et al*, 1997; Curran *et al*, 1996). Therefore it is possible that a conformational change in the trimer structure compared to the monomer prevents the nuclear localisation signal from being recognised.

3 A model for RNA Replication

During viral transcription, the polymerase complex responds to signals telling it to generate discrete leader and mRNAs. In contrast, during replication, the polymerase must ignore these signals and generate genome-length positive strand RNA. This RNA is then replicated to form the genomic minus-strand RNA. An accepted model to explain the switch between transcription and replication states that newly synthesized NP binds the nascent RNA thus preventing recognition of the termination signals. This model is supported by work on VSV where leader sequence was shown to be selectively encapsidated by NP (Blumberg *et al*, 1983).

The model is attractive because immediately after virus entry, transcription would be favoured over replication. However, at later times in infection, when the NP concentration has increased, replication would be favoured.

A recent study on SeV P protein suggested that only trimeric P could interact with L thus forming the active polymerase complex which could interact with the RNP template via the C-terminal tails of the P trimer (Curran *et al*, 1997). Transcription or replication of the genome (or anti-genome) template would rely on the polymerase complex being able to traverse the length of the template (Fig.53, Panel A). These authors determined that only 2 C-terminal tails of the trimer were necessary for stable interaction with the RNP but all 3 were necessary for viral RNA synthesis. Therefore, the third C-terminal leg of the trimer was proposed to be involved in P migration along the length of the RNP template. Simultaneous breaking and reforming of contacts between the third C-terminal leg and the RNP, such that two legs are always in contact with the RNP, would lead to polymerase movement along the template by a rotation mechanism.

The overall replication process can be broadly split into 3 mechanistically linked processes; initiation of RNA synthesis, initiation of RNA encapsidation and elongation of encapsidation. Both P and V may have active roles in both the encapsidation and elongation steps of genome replication. Alternatively, V may play a role in replication

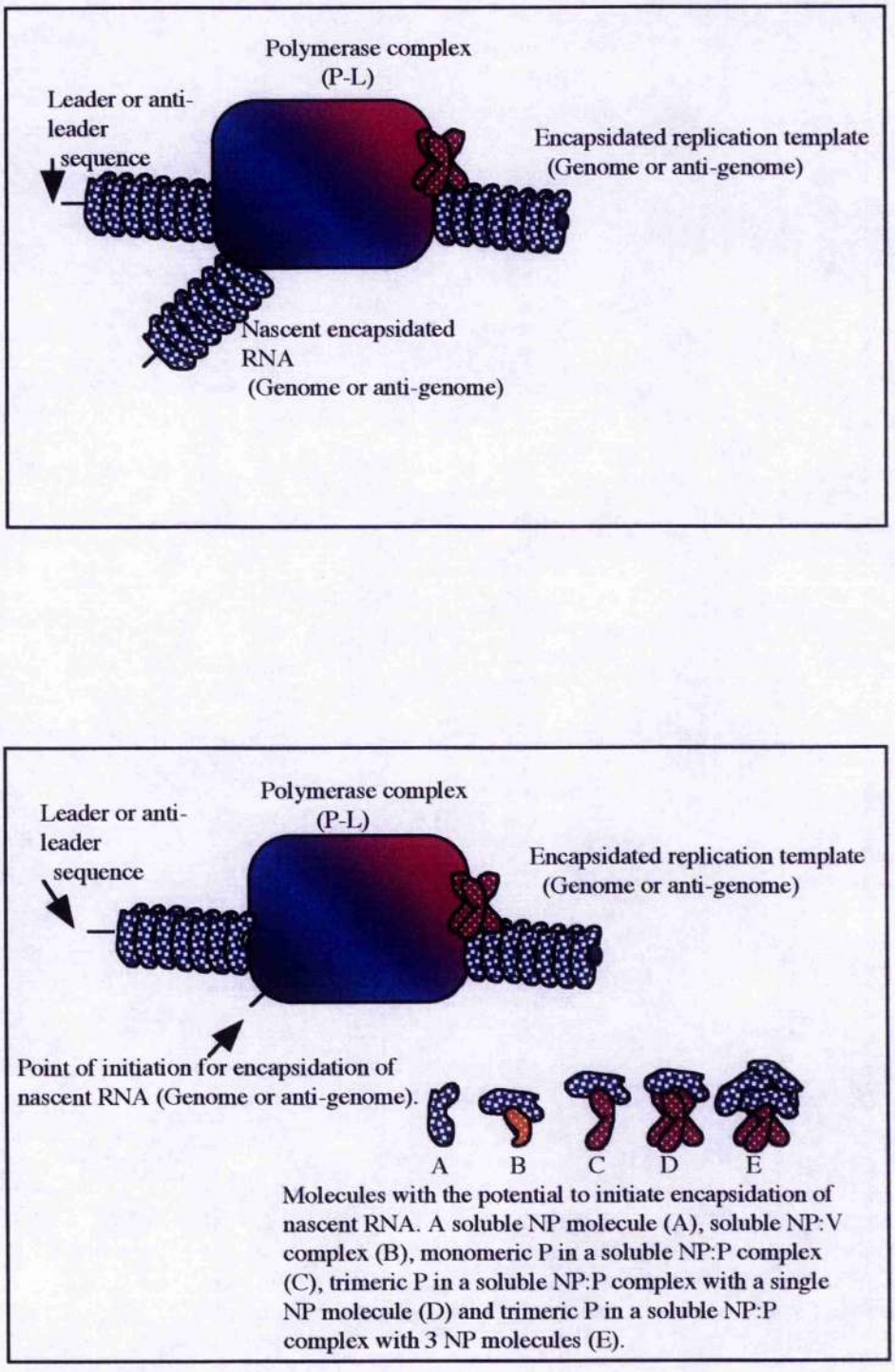

Fig. 53 Initiation of RNA encapsidation during replication

inhibition and/or viral pathogenesis. The possible role(s) of these proteins in the steps of genome replication are discussed below.

3.1 Initiation of RNA synthesis

Little is known about the mechanism which initiates paramyxovirus RNA synthesis. The polymerase complex (P/L) is thought to bind at the 3' terminus of the RNA template, which acts as the sole polymerase entry site, or promoter, for the initiation of RNA synthesis during both transcription and replication. The leader (and anti-leader) sequences have therefore been assumed to contain specific recognition sequences for the polymerase, but the mechanism of polymerase targeting to the precise 3' terminus has yet to be defined.

3.2 Initiation of RNA encapsidation

In the absence of encapsidation, the leader sequence is lost, resulting in polymerase reinitiation at the junction between the leader and the first protein-encoding gene. At this point the polymerase is committed to transcriptase mode, leading to the generation of discrete viral mRNAs. Therefore one of the most important events during viral replication, is the initiation of nascent RNA encapsidation.

A model for the initiation of RNA encapsidation is given in Fig.53 (Panel B), and suggests a number of candidates which could theoretically deliver the initial NP molecule to the 5' terminus of the nascent genomic RNA. A soluble NP molecule has the potential to bind at the 5' end of the nascent RNA genome thus initiating encapsidation. However, it is unclear how this molecule could direct its own binding to the precise 5' end of the nascent RNA and therefore although possible, this does not seem the most feasible candidate.

A soluble NP:V complex could potentially be targeted to the 5' end of the nascent genome by V binding directly to the nascent RNA, thus anchoring the first NP molecule at the end of the genome. Once the first NP molecule is bound to the RNA, the elongation process can begin. It is possible that V would be released from the

complex by the conformational change in NP proposed above, thus allowing V to bind a further NP molecule.

NP:P complexes could play a similar role in the initiation of nascent RNA encapsidation but may not dissociate from the template after delivery of the NP molecule. If the P trimer remained bound to the 5' end of the nascent genomic RNA, a further L protein could be recruited to the promoter site for further rounds of transcription/replication of the nascent RNP.

3.3 Elongation of RNA encapsidation

Once initiated, a constant supply of encapsidation substrate must be available for the elongation process which ensures the entire genome/anti-genome is encapsidated. A schematic diagram depicting the delivery of soluble NP during the elongation step of RNA encapsidation, is shown in Fig. 54. The mechanism described for the initiation of RNA encapsidation can also be used during the elongation phase. The NP:P and NP:V complexes described above could then play chaperoning roles for delivery of soluble NP to the nascent nucleocapsid, allowing P or V proteins to dissociate once delivery is complete. Fig.54 Panel A, shows the NP:V complex delivering a soluble NP molecule to the elongating nucleocapsid. Once delivered, V would dissociate from the polymerised chain due to the proposed conformational change in NP and would be free to re-associate with further soluble NP molecules, thus continuing the elongation process.

NP:P complexes may use the same mechanism to deliver NP to the growing nucleocapsid as outlined for NP:V. Once the NP molecule(s) have been delivered, P may dissociate from the nascent nucleocapsid to bind further soluble NP molecules (Fig.54, Panels B and C). P may, however, remain in association with the nucleocapsid to target L protein to the nascent RNP in preparation for virion assembly and release (Fig.54, Panel D). Alternatively, P may target L onto the nucleocapsid for further rounds of transcription / replication as mentioned above. However, since the only polymerase entry site (promoter) is at the 3' end of the genome, any new P-L complexes

Fig. 54 Delivery of soluble NP to nascent RNA for encapsidation.

Schematic diagram depicting potential mechanisms for the delivery of soluble NP to the nascent RNA during the elongation step of RNA encapsidation. Panel A shows soluble NP:V complexes as the substrate for encapsidation. NP is held in a soluble NP:V complex until it is delivered to the growing nucleocapsid chain. Once bound to the nascent nucleocapsid, V dissociates, allowing it to complex with a free NP molecule thus forming another soluble NP:V complex.

Panel B shows monomeric P in soluble NP:P complexes as the substrate for encapsidation. By the same mechanism as Panel A, soluble NP:P complexes can be used to deliver NP to the nascent nucleocapsid, leading to dissociation of P. The P molecule is now free to bind more soluble NP thus forming a another soluble NP:P complex.

Panel C shows trimeric P in soluble NP:P complexes as the substrate for encapsidation. The NP:P complex is depicted as trimeric P binding one NP molecule, but trimeric P could have equally been depicted as binding three NP molecules. Again the delivery mechanism shown, leads to dissociation of the trimeric P after delivery of the NP molecule(s) to the growing nucleocapsid chain, allowing trimeric P to bind further NP molecules.

In Panel D, P trimers are depicted as binding 3 NP molecules in a soluble NP:P complex which acts as the encapsidation substrate. Once the NP molecules are delivered, P is depicted as staying bound to the nascent nucleocapsid. By remaining bound, P can recruit the polymerase protein (L) to form an active polymerase complex (P-L) in preparation for virion assemble and release.

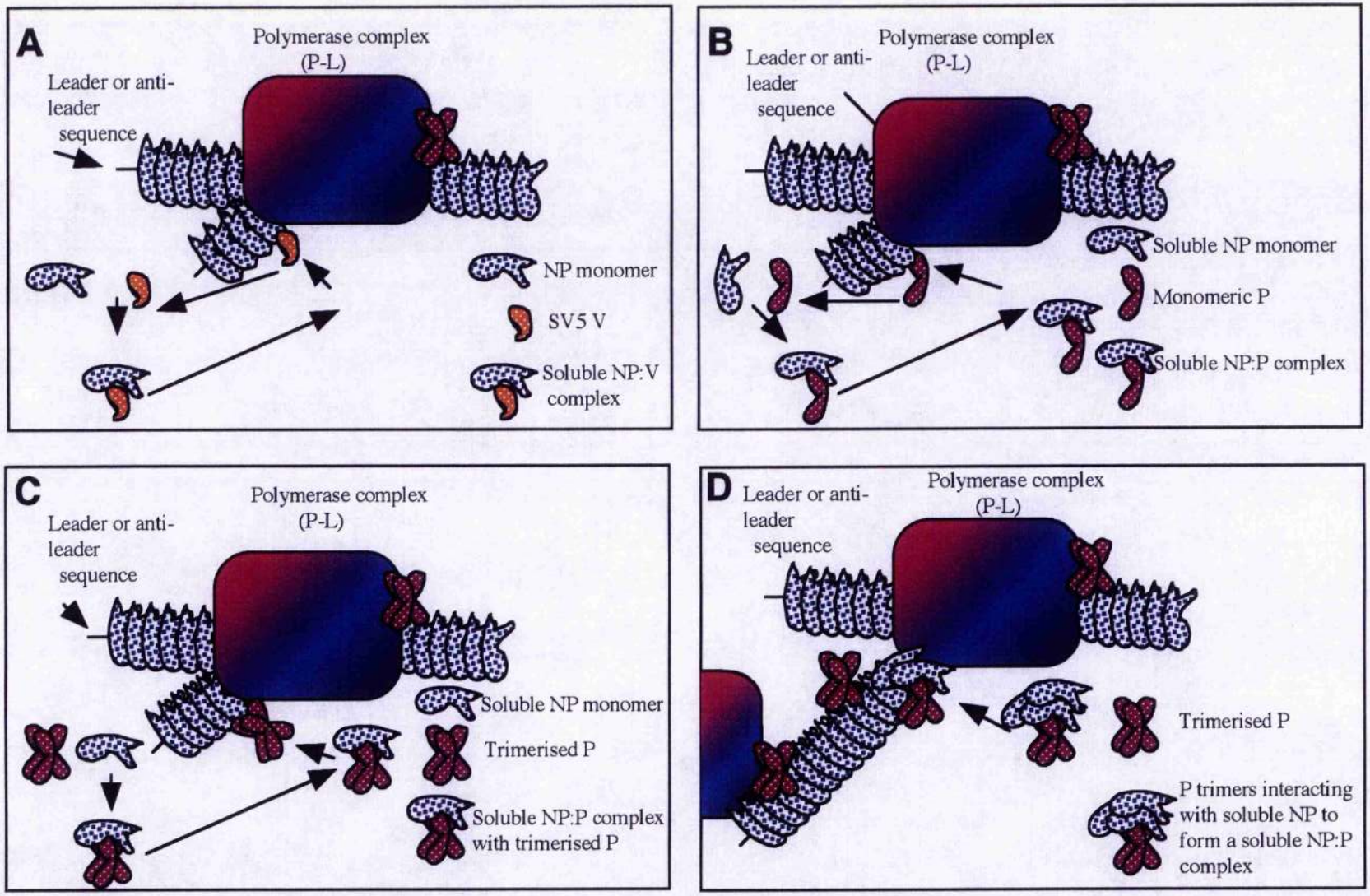

Fig. 54 Delivery of soluble NP to nascent RNA for encapsidation

formed along the length of the RNP would have to dissociate from the nucleocapsid and re-associate at the promoter region (Fig.54, Panel D).

3.4 Possible roles for V in replication

The V and W proteins of SeV have been shown to both inhibit genome replication and also form complexes with soluble NP (Horikami *et al*, 1996) and is in agreement with the results presented in this thesis (Randall and Bermingham, 1996). A recent study on SeV suggested that monomeric P, as well as V and W proteins, could bind soluble NP but these soluble complexes were subsequently unable to form active assembly complexes for encapsidation (Curran *et al*, 1997). This suggests that SV5 NP:V complexes may not be active in RNP assembly as outlined thus far. Even if the NP:V complex plays no direct role in encapsidation, a role in the inhibition of genome replication can be postulated.

V may compete with P for the common binding site on NP thus delaying encapsidation by depleting the pool of soluble NP available for encapsidation and would result in inhibition of genome replication. NP:V complex formation would also prevent both NP:NP homopolymer and NP:P heteropolymer formation in the absence of RNA replication.

However, the possible roles ascribed to V for inhibition of genome replication or initiation of encapsidation may be auxiliary functions to the fundamental mechanism of paramyxovirus replication. He *et al* (1997) successfully rescued a full-length SV5 anti-genome clone by supplying only NP, P and L protein (and not V protein) suggesting that the synthetic RNA had undergone at least one round of replication in the absence of V. (After the initial round of replication, the resultant negative-sense RNA could be used as a transcription template, thus generating V mRNA and therefore V protein.) This suggests that V is non-essential for SV5 replication and supports similar findings from both SeV (Kato *et al*, 1997a; Delenda *et al*, 1997)and MeV (Schneider *et al*, 1997).

In the two recent reports on SeV, full-length recombinant viruses were rescued from cDNA clones which did not encode a V protein, thus demonstrating that V was not an essential factor for SeV replication (Delenda *et al*, 1997; Kato *et al*, 1997a). The latter

report found that the recombinant virus displayed augmented gene expression and cytopathogenicity *in vitro*, but was strongly attenuated in mice.

A further study by Kato *et al* (1997b) generated a recombinant SeV expressing a C-terminally deleted V protein ($V_{\Delta C}$) for comparison to the V-minus and wild-type SeV. $V_{\Delta C}$ virus was able to grow in both tissue culture and in eggs, strengthening further the notion that V is a non-essential gene product. However, expression of the N-terminal domain of V protein alone was sufficient to convert the augmented phenotype, due to total deletion of V protein, to the phenotype characteristic of the wild-type SeV. The mechanism for maintaining the normal phenotype, probably involving the N-terminus of NP, remains to be elucidated, but may involve the NP chaperoning function associated with this region of V.

The $V_{\Delta C}$ virus, like the V-minus virus, was found to be much less pathogenic *in vivo* than wild-type SeV, supporting the notion that V protein encodes a luxury function for *in vivo* pathogenesis (Kato *et al*, 1997). A similar basis for attenuation was suggested for both the V-minus and $V_{\Delta C}$ viruses by postulating that the C-terminal half of the V protein contained the element important for SeV pathogenesis.

Perhaps V is necessary to evade host defence mechanisms such as natural killer (NK) cells and induction of interferons (Anderson, 1982; Muller *et al*, 1994) but these evasion pathways have yet to be elucidated. Recent work in this laboroatory has shown that SV5 V protein specifically interact with a cellular protein of 150 kDa, which has, as yet, not been identified (Precious *et al*, 1995). It would be interesting to see if this unidentified protein played any role in triggering the anti-viral response in the infected cell and if so, was this role in any way impaired by the binding of V.

In the *Rubulaviruses* , the V gene is encoded by the genome, while in the *Parainfluenzaviruses* and *Morbilliviruses* encode the P gene and generate V protein by mRNA editing. Also, during an SV5 infection, more V mRNA than P mRNA is transcribed (B. Precious and R.E. Randall, unpublished observations) suggesting an important role for V in the virus life-cycle.

4 The future for negative-sense RNA viruses

The ability to genetically alter negative-strand RNA viruses has enhanced this field of virology and may have a major influence on future developments of structure-function analysis, vaccine development and the use of virus vectors. Firstly, structure-function studies of individual genes are now possible, in the context of an infectious virus, for a number of negative-strand RNA virus families, including a number of medically important viruses (e.g. MeV, RSV, hPIV3 and influenza viruses). In the recent past, viral genes were studied in isolation by cloning and expressing them in different systems. Current approaches favour questioning the roles played by viral genes and gene products when interacting with host cell components and the host in general. This can be best done by studying genetically defined viruses and subjecting them to direct mutational analysis of individual genes, promoter elements or other non-coding sequences. These recombinant viruses can then be subjected to biochemical analysis and studies on replication, both *in vitro* and *in vivo*.

Secondly, genetically engineered negative-strand RNA viruses could become live virus vaccine candidates. The tools are now available to design a new generation of vaccines for the medically important negative-strand RNA viruses, such as RSV and parainfluenza viruses, where previous approaches have failed to result in acceptable vaccine candidates.

Finally, negative-strand RNA viruses may become useful vectors for the expression of foreign genes. Recombinant rabies virus (Mebatsion *et al*, 1996), VSV (Schnell *et al*, 1996) and SeV (Hasan *et al*, 1997) have already been used to express additional protein sequences or foreign genes. The major advantage in using negative-strand RNA viruses as vectors (or vaccines) is that these viruses do not go through a DNA phase and thus cannot transform cells by integrating their genetic information into the host genome. Furthermore, homologous recombination has never been observed in any of these negative-strand RNA viruses. Therefore, contaminating virus should not be generated when replication-incompetent viral constructs are grown in complementing cell lines.

The negative-strand RNA virus vectors are therefore safer, and thus more attractive, than their DNA counterparts.

The solution to may of the issues discussed in this thesis will depend on the continued development of novel strategies to study viruses and their interaction with the host cell, leading us to a better understanding of the natural world.

Appendix 2

i) Primers used in the construction of pUCSV5CAT as shown in Fig. 17

1) SV5 Leader region forward primer :-

Sac1
5' ATC TTC **GAG CTC** TTC CAC CAG GGG AAA ATG TGG TGA CTC AAA TCA TCG AAG ACC CTC GAG ATT ACA TAG GTC CGG 3'

Forward primer in PCR reaction to generate pUCSV5CAT from pUCSV5TKCAT.

2) Beginning of CAT ORF reverse primer

Bstl1071
5' CAA CGG TG**G TAT AC**C CAG TGA TTT TTT TCT CCA TTT TTG TGT CGG GGA CGA AAA ATT GC 3'

Reverse primer in PCR reaction to generate pUCSV5CAT from pUCSV5TKCAT.

ii) Primers used in the construction of pGEM-BLNP as shown in Fig. 20

1) NP forward primer :-

BamH1 |> Beginning of NP ORF
5' TAG CCT **GGA TCC** *ATG TCA TCC GTC CTT AAA GCA* 3'

Forward primer in PCR reaction for pGEM-BLNP from pBR322NP and vRNA

2) NP reverse primer :-

HindIII |< End of NP ORF
5' TAG CCT **AAG CTT** *CTA GAT GCT AAG ATC ACC CAG TGC* 3'

Reverse primer in PCR reaction for pGEM-BLNP from pBR322NP and vRNA

iii) Primers used in the construction of pGEMβGlobinNP as shown in Fig. 25.

1) βGlobin forward primer :-

EcoR1
5' TAC ATA **GAA TTC** ACA CTT GCT TTT GAC ACA ACT GTG TTT ACT TGC AAT CCC CCA AAA CAG ACA GA*A TG* 3'

Forward PCR primer used to generate pGEMβGlobinNP from pGEMBLNP where NP ORF initiation ATG is shown in Italics.

2) βGlobin reverse primer :-

Nde1
5' ACT *ATC GCT* ***CAT ATG*** *CTT TAA GCA CGG ATG ACA T* TC TGT CTG TTT TGG 3'

Reverse PCR primer used to generate pGEMβGlobinNP from pGEMBLNP where NP ORF sequences are shown in Italics.

iv) Primers used in the construction of pPANHAN as shown in Fig. 30.

1) pPANHAN forward primer :-

HindIII
5' GAA TCA GAA TAC GTA T**AA GCT T**AA AGA AGA GGA T 3'

Forward primer in PCR reaction to generate pPANHAN from pT7.1.

2) pPANHAN reverse primer :-

Sal I
5' GCG **GTC GAC** GCT GAG TAC CAA GGG GAA 3'

Reverse primer in PCR reaction to generate pPANHAN from pT7.1.

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